Bioprocess Studies of Biomass and Recombinant Protein Production by the Methylotrophic Yeast *Pichia Pastoris*

A thesis submitted to University College of London
for the degree of Doctor of Philosophy

By Patrick Randone

2014

Department of Biochemical Engineering
University College of London (UCL)
Gower Street
London, WC1E 6BT
United Kingdom

‘ I, Patrick Randone confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Signed.....

Date.....13.12.2013.....

Acknowledgements

I would like to thank my supervisors Dr. D. Nesbeth and Dr. E. Keshavarz-Moore for their assistance all along this project and for the support given. I would like also to thank Dr. A. Lopes, Dr. G. Mannall, S. Woodhouse and A. Wei for their technical support.

Abstract

Pichia pastoris (*P. pastoris*) expression systems are gaining increased interest in industry due to their ability to achieve high cell densities and production levels. This thesis aims to characterise the following critical elements of *P. pastoris* upstream bioprocessing: cell growth, cell productivity and bioprocess monitoring. Methanol is the principle carbon source and gene expression induction agent in most *P. pastoris* fermentation strategies. Monitoring of methanol levels during fermentation enables cell growth and productivity to be optimised, whilst methanol toxicity is avoided. A novel approach to at-line methanol monitoring was investigated, seeking to exploit the chromogenic reactivity of dehydroascorbic acid (DHAA) with methanol. Recombinant variants of the human reporter enzyme, placental alkaline phosphatase (PLAP) were used as a model fermentation product, and the performance of two mechanistically distinct commercial assays was compared for their applicability to high cell density process streams. In total four new *P. pastoris* strains expressing PLAP variants were successfully created and the impact of methanol carbon source feed strategies on the strain performance investigated in terms of growth rate and recombinant protein production. A 2-fold increase in rate of methanol feed resulted in a final biomass increase of about 40% and in a volumetric productivity decrease of about 75%. A novel method, combining phases of low and high methanol feed rates, matched but did not exceed conventional methanol feed rate strategies.

Table of content

List of figures

List of tables

Abbreviations and symbols

(v/v)	volume/volume
$2H_2O$	Dihydrate
$5H_2O$	Pentahydrate
$7H_2O$	Heptahydrate
αMF	α mating factor from *S. cerevisiae*
αPLAP	Yeast secretion signal (α-MF) - PLAP
β-gal	β-galactosidase
β-ME	β-mercaptoethanol
µ	micro / growth rate
μ_{max}	Maximum growth rate
Amp^r	Ampicillin resistance
AOD1	*Candida boidinii* methanol oxidase
AOX1/2	Alcohol oxidase 1/2
AP	Alkaline phosphatase
ATP	Adenosine tri-phosphate
ATPSs	Aqueous two-phase systems
AUG1/2	
B	Biotin
BMGY	Buffered minimal glycerol-complex medium
BMMY	Buffered minimal methanol-complex medium
bp	base pairs
BSA	Bovine Serum Albumin
BSM	Defined basal salts media
C	Carbon
Ca	Calcium
cAMP	Cyclic adenosine mono-phosphate
CAT	Catalase
CFM	Conventional methanol feed rate method
CH_3OH	Methanol
CIP	Calf intestinal alkaline phosphatase
Cl	Chlorine
Cu	Copper
D	D-glucose
DAM	Desferrioxamine mesylate
dATP	Deoxyadenosine tri-phosphate
DAS	Dihydroxyacetone synthase
dCTP	Deoxycytidine tri-phosphate
DCW	Dry cell weight
ddH_2O	Double-distilled water
dGTP	Deoxyguanosine tri-phosphate
DHA	Dihydroxy acetone
DHAA	Dehydroascorbic acid

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides tri-phosphate
DO	Dissolved oxygen
DoE	Design of Experiments
DOT	Dissolved oxygen tension
dTTP	Thymidine tri-phosphate
E.	*Escherichia*
EBA	Expanded bed adsorption
EDTA	Ethylenediaminetetracetic acid
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FDH	Formate dehydrogenase
Fe	Iron
fg	Femtogram
FGH	S-formylgluthatione hydrolase
FLD	Formaldehyde dehydrogenase
FM22	Fermentation medium 22
G418	Geneticin
GAP	Glyceraldehyde-3-phosphate
GCAP	Germ-cells alkaline phosphatase
GFP	Green fluorescent protein
GRAS	Generally regarded as safe
GSH	Gluthatione
GY	Glycerol
H.	*Hansenula*
hPLAP	Human secretion signal - PLAP
H_2O	Water/hydrate
H_2O_2	Water hydroxyde
HeLa	Immortal cell line derived from cervical cancer in 1951
HFM	High methanol feed rate method
HIC	Hydrophobic interaction chromatography
His^+/HIS4	*Pichia pastoris* wild-type gene coding for histidinol dehydrogenase
His^-/his/his4	*Pichia pastoris* strains missing the histidinol dehydrogenase gene
HPLC	High-performance/pressure liquid chromatography
HRP	Horseradish peroxidase
HSA	Human Serum Albumin
K_2HPO_4	Potassium phosphate dibasic / Dipotassium phosphate
Kan^r	Kanamycin resistance
kDa	kilo Daltons, protein size unit
KH_2PO_4	Potassium phosphate monobasic / Potassium phosphate
K_I	Inhibitor constant / dissociation constant
KJ	Kilo Joule
K_M	Michaelis-Menten constant

KOH	Potassium hydroxide
K_S	Substrate constant
IAP	Intestinal alkaline phosphatase
IMRC	Innovative Manufacturing Research Centre
LB	Lysogeny broth (also known as Luria broth, Lennox broth, or Luria Bertani medium)
M	Molarity – moles/litre ("molar")
M (10x)	Methanol
m-RNA	messenger RNA
MCB	Master Cell Bank
MCS	Multiple Cloning Site
MD	Minimal D-glucose medium
MFS	Methylformate synthase
Mg	Magnesium
$MgCl_2$	Magnesium Chloride
ml/h/L	milli litres per hours per litre initial fermentation volume
MLFB	Methanol-limited fed-batch
MM	Minimal methanol medium
Mn	Manganese
MOD1/2 (AUG1/2)	Alcohol oxidase genes in *P. methanolica*
mol	moles
MOX	Methanol oxidase
mS cm^{-1}	Ionic conductivity
Mut	Methanol utilisation
$Mut^{+/s/-}$	Methanol utilisation positive/slow/negative
Mxr1p	Zinc finger protein that regulates AOX1 activity
MW	Molecular weight
N	Nitrogen
NaAc	Sodium acetate
NaCl	Sodium chloride, salt
NAD+/NADH	Nicotinamide adenine dinucleotide
NaH_2PO_4	Sodium phosphate
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
NH_4SO_4	Ammonium sulphate
NIRS	Near infrared refractance spectroscopy
OD	Optical density
OLFB	Oxygen-limited fed-batch
P.	*Pichia*
P	Phosphate
P_{AOX1}/ P_{gene}	AOX1 promoter / gene promoter
PBS buffer	Phosphate buffered saline buffer
PCR	Polymerase Chain Reaction
pDNA	plasmid DNA
PEG	Polyethylene glycol
PEP4	Encodes for protease A in *P. pastoris*

PEX8	Intraperoxisomal peroxin, required for protein transport into peroxisomes
PID	Proportional integral derivative
PLAP	Placental alkaline phosphatase
PMSF	Phenylmethylsulfonyl fluoride
PRB1	Encodes for protease B in *P. pastoris*
PTM1	*Pichia* Trace Metals (1L preparation)
QB	Quanti-Blue
RCT	Research Corporation Technologies
RNA	Ribonucleic acid
RO	Reverse Osmosis
Rpm	Rotations per minute
RT	Room temperature
S.	*Saccharomyces*
S	Sulfur / Substrate
S-HMG	S-hydroxymethylglutathione
scFv	Single-chain variable fragment
SEAP	Secreted Embryonic Alkaline Phosphatase
SEC HSA	Secreted Human Serum Albumin
Sec61p	Polytopic membrane protein
SIA	Sequential injection analysis
SIP	Sterilise in place
TBE	Tris-Borate-EDTA
TE buffer	Tris-EDTA buffer
TFBS	Transcription factor-binding sites
TLFB	Temperature-limited fed-batch
TNSAP	Tissue non-specific alkaline phosphatase
UAS	Upstream activation region
URA3	Orotidine 5-phosphate decarboxylase
URS	Upstream repression sequence
vvm	Volume per volume per minute
WCB	Working cell bank
WCW	Wet cell weight
WT	Wild-type
Xu_5P	Xylulose-5-phosphate
YNB	Yeast nitrogen base
YPD	Rich, undefined media – yeast extract, peptone, D-glucose
YPDS	YPD + 1.2M Sorbitol
yscCo	Cobalt-dependent aminopeptidase
Zeo^r	Zeocin resistance
Zn	Zinc

1. Introduction

1.1. Project significance

Pichia pastoris is widely known as a host for expression of recombinant proteins. Major advantages of this microorganism are its ability to express proteins under the control of the strong alcohol oxidase (AOX1) promoter to grams per litre levels in simple salt media and to be able to grow to very high cell densities. Other advantages are its ability to do post-translational modifications similar to higher eukaryotes and to be able to straightforwardly accept foreign DNA (Macauley-Patrick *et al.*, 2005; Krainer *et al.*, 2012).

On the other hand, there are some drawbacks of this expression system. First, downstream processing of high cell density cultures is laborious and usually results in high product losses when it comes to solid/liquid separation. A lot of work is still required in order to fully exploit this microorganisms to its best. For instance, performance is usually protein/product dependant, and also the choice of the *Pichia pastoris* strain to use require multiple test for the best methanol utilisation (Mut) phenotype (positive, slow, or negative) to use. Furthermore, methanol (carbon source and inducer) may cause safety issues, especially when used in large amounts (Macauley-Patrick *et al*.,2005; Ohi *et al*., 1994; Krainer *et al.*, 2012).

Within this project, some research questions were attempted to be answered. This was done by drawing some primary objectives (creating new *Pichia pastoris* strains and increasing productivity of a reporter protein) as well as secondary objectives aimed to reduce costs (in-house made PLAP standard), increase safety (methanol detection assay),

and reduce time (multiple shake flask comparison of different strains before scaling up to bioreactor experiments). Therefore, the information found in the literature was brought together to draw objectives that were experimentally tested and verified.

1.2. Bioprocess Host Cells

For many generations the yeast *Saccharomyces cerevisiae* has been used in baking and brewing. In 1969 the first methylotrophic yeast was isolated, and since 1970 other species have started to gain industrial interest (Yurimoto & Sakai, 2009) as bioprocess host cells, which ideally would be able to easily accept foreign DNA to produce high titres of a desired recombinant protein in a robust, stable, and scalable system. Methylotrophic species are able to use methanol as a sole source of carbon and produce high titres of recombinant proteins, of these the most commonly used strains are: *Pichia pastoris*, *Pichia angusta* (also known as *Hansenula polymorpha*), *Pichia methanolica*, and *Candida boidinii* (Houard *et al.*, 2002). Decaying plant materials have been the main source used for the isolation of most methylotrophic yeasts. *Pichia pastoris,* for example, was first isolated from an exudate of oak (Van der Klei *et al*., 2006). As methanol is released during hydrolysis of pectin, which is an abundant plant constituent, decaying vegetal materials are natural sites for methylotrophic yeasts (Van der Klei *et al.*, 2006).

Being a relatively cheap substrate, methanol was increasingly gaining interest among the scientific community for biotechnological, biochemical, and chemical processes (Yurimoto & Sakai, 2009). Thus, high-cell-density cultivation methods took over and a number of methanol-induced genes involved in the metabolism of methanol

have been isolated and studied. Furthermore, high cell density and gene technology methods combined with cheap synthetic salt-based media made it possible and economically valuable to produce large numbers of heterologous proteins (http://faculty.kgi.edu/cregg) such as enzymes, antibodies, cytokines, plasma proteins, and hormones (Gellissen, 2000).

Just like *E. coli*, *P. pastoris* can be engineered relatively easily, grown in simple media and scaled-up. Furthermore, as with all eukaryotic cells (Figure 1.1), they have the capability of trafficking proteins and enzymes in order to process and glycosylate them. Yeasts offer the opportunity for pharmaceutical applications (Hartner & Glieder, 2006) as their products are generally regarded as safe (GRAS status).

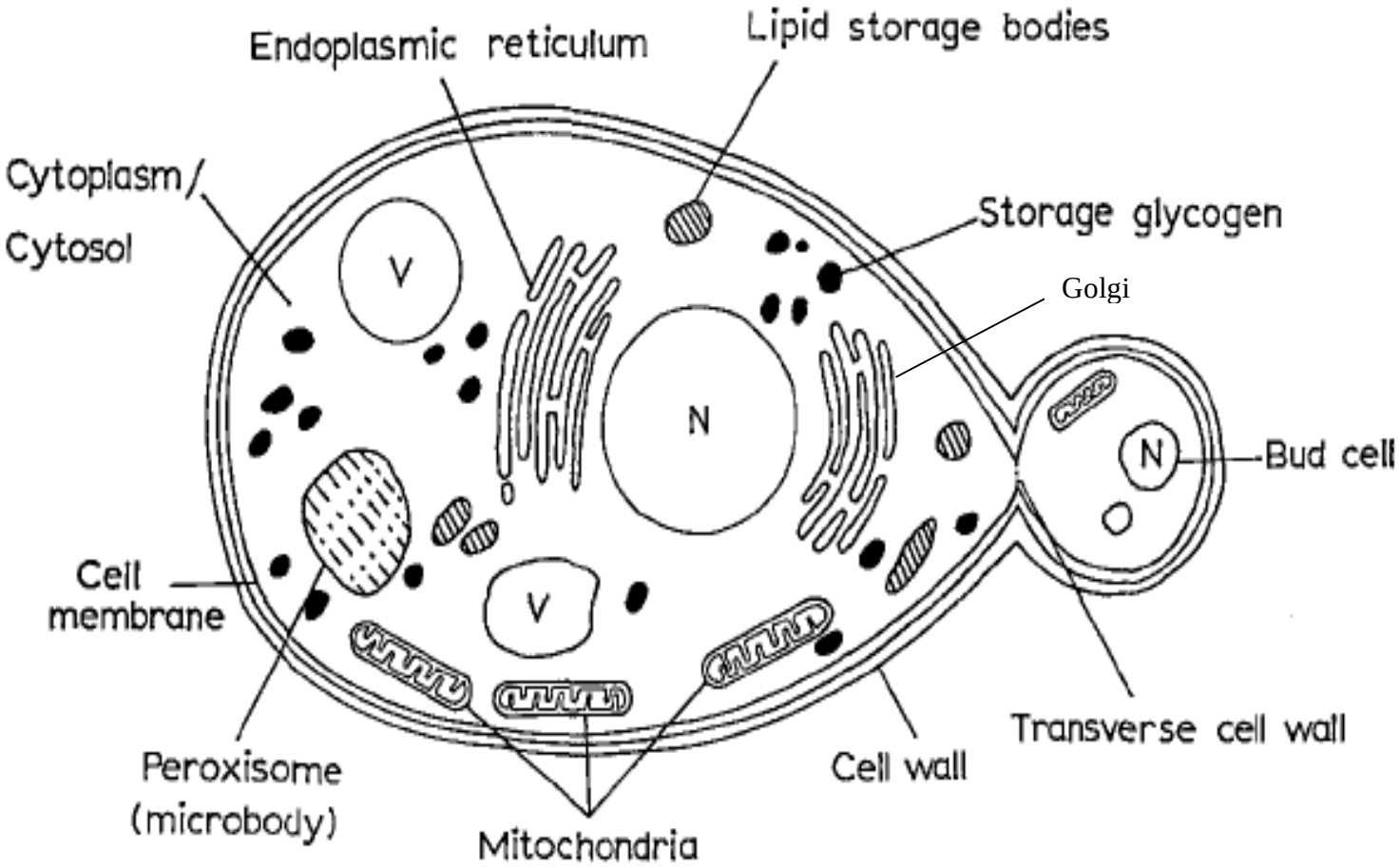


Figure 1.1: Typical yeast cell
Idealised representation of a typical yeast cell. N: nucleus; V: vacuole (Ratledge, 1991). In *Pichia pastoris*, ordered Golgi stacks are located next to stable ER sites. A typical *P. Pastoris* cell contains 2-5 ER-Golgi units (Papanikou & Glick, 2009).

Recently, engineered strains of human-like *P. pastoris* have been developed. As a consequence, methylotrophic yeasts are gaining new markets for the production of therapeutic proteins, which were mainly produced by *E. coli, S. cerevisiae* and mammalian cell lines thus far (Hartner & Glieder, 2006). This is facilitated by the fact that protein folding in yeasts happen through cellular structures and mechanisms that are also found in higher eukaryotes (Yurimoto & Sakai, 2009). Furthermore, the easy to achieve high yield productions obtained with *Pichia pastoris* fosters research in this field, which is gaining consensus as a universally-recognised solution to overproduce recombinant proteins for applications ranging from structural genomics to biopharmaceutical manufacture (Holmes *et al.*, 2009). Another advantage of *Pichia pastoris* as a host for recombinant protein production compared to other cell lines, is the fact it offers a more straightforward downstream process, which for example, in cases of proteins having low production titres (e.g. alkaline phosphatases) is often preferred to the better cost efficiency of *E. coli* cells (Heimo *et al*., 1997).

Only a small number of yeasts (Table 1.1) are able to use methanol as a sole source of carbon. Unlike *S. cerevisiae*, methylotrophic yeasts belong to the Crabtree negative group (Van der Klei *et al*., 2006). Under aerobic conditions ethanol is produced at a very low titre by Crabtree negative yeasts, enabling very high cell densities and production yields. In addition, some of these yeasts are able to produce recombinant proteins up to several grams per litre. High production titres of protein are tightly related to promoters of genes involved in the methanol utilisation (Mut) pathway (Van Urk *et al*., 1990; Sivaprakasam *et al.*, 2011).

A list of isolated genes and promoters can be found in Table 1.3. Alcohol oxidase I (AOX1) is the most widely used, as well as one of the strongest and most tightly controlled in nature (Van der Klei *et al.*, 2006). This promoter is naturally found in the *Pichia pastoris* genome, whose total estimated size is 9.7 million base pairs organised into four chromosomes (De Schutter *et al.*, 2009).

Table 1.1: Yeast growth in different carbon sources

Yeast able or supposed able to grow on methanol and ability ferment or to use D-glucose and glycerol as a sole source of carbon for growth (Barnett *et al.*, 1990; Barnett *et al.*, 2000).

Yeast	D-glucose fermentation	D-glucose growth	Glycerol growth	Methanol Growth
Candida boidinii	+	+	+, D	+
Candida cellulolytica	+	+	+	+
Candida drosophilae	-	+	W	?
Candida cariosilignicola	D	+	+	+
Candida endomychidarum **	?	?	+	W
Candida maris	None	+	+, D	+
Candida methanolophaga	+	+	+	+
Candida methanosorbosa	+	+	+	+
Candida methylica	+	+	+	+
Candida nanospora	+	+	+	+
Candida nemodendra	None	+	+	+
Candida nitratophila	D	+	+	+
Candida ooitensis	+	+	+	+
Candida ovalis	+	+	+	+
Candida pignaliae	+	+	+	+
Candida pinus	D	+	+	+
Candida sonorensis	+	+	D	+
Candida succiphila	+	+	+	+
Dipodascus albidus	None	+	+	?
Dipodascus ambrosiae	None	+	+	?
Dipodascus armillariae	None	+	+	?
Dipodascus australiensis	None	+	+	?
Cyniclomyces guttulatus	+	-	?	?
Hansenula ofunaensis	None	+	+	+
Galactomyces citri-aurantii	-	+	+	?
Oosporidium margaritiferum	-	+	v	?
Pichia angusta	+	+	+	v
Pichia arabinofermentas	D	+	+	v
Pichia capsulata	+	+	+	v
Pichia ciferrii	+	+	+	v
Pichia finlandica	None	+	+	+
Pichia galeiformis	v	+	v	+
Pichia glucozyma	+, D	+	+	+
Pichia henricii	D	+	+	+
Pichia kodamae	+	+	+	D
Pichia lachancei	+	+	+	?
Pichia methanolica	+	+	+, D	+
Pichia methylovora	D	+	+	+
Pichia minuta	v	+	+, D	+
Pichia naganishii	+	+	+	+
Pichia ofunaensis	-	+	+	+
Pichia pastoris	+	+	+	+
Pichia philodendra	None	+	+	+
Pichia pini	v	+	+	v
Pichia trehalophila	+	+	+	+
Trichosporiella flavificans	W	+	+	?

Legend: + positive; - negative; D delayed longer than 7 days; W weak utilization; v variable utilisation; None no fermentation with any carbon source; ? result not known; ** Suh *et al.*, 2005.

1.3. Advantages of Pichia pastoris recombinant protein production platform

1.3.1. *Pichia pastoris* Biology

1.3.1.1. Metabolism and enzymes involved

All methylotrophic yeasts have a distinctive Methanol utilisation (Mut) pathway (Figure 1.2) that is regulated at the transcriptional level. Peroxisomes control the first steps of this pathway. Thus, these organelles may account up to 80% of the cytoplasmic space after methanol induction (Yurimoto, 2009). First, alcohol oxidase (*AOX*) oxidise methanol to formaldehyde and hydrogen peroxide. Next, catalase (*CAT*) breaks down the toxic H_2O_2 into oxygen and water. Formaldehyde is assimilated by condensation with xylulose-5-phosphate (Xu_5P) or oxidised by two successive reactions (dissimilation pathway). Xu_5P and formaldehyde are converted into C_3-compounds dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (GAP) by dihydroxyacetone synthase (DAS). Further metabolic reactions (dissimilation pathway) occur in the cytosol, where formaldehyde spontaneously reacts with glutathione to form S-hydroxymethylglutathione (S-HMG). Glutathione (GSH) - and NAD^+-dependent formaldehyde dehydrogenase (*FLD*) and NAD^+-dependent formate dehydrogenase (*FDH*) oxidise in two consecutive reactions S-HMG to carbon dioxide. NADH generated in these reactions is believed to be exploited for growth on methanol. Furthermore, S-formylglutathione hydrolase (*FGH*) catalyses both the detoxification of formaldehyde and the regeneration of glutathione (Babel & Hofmann, 1982; Yurimoto, 2009).

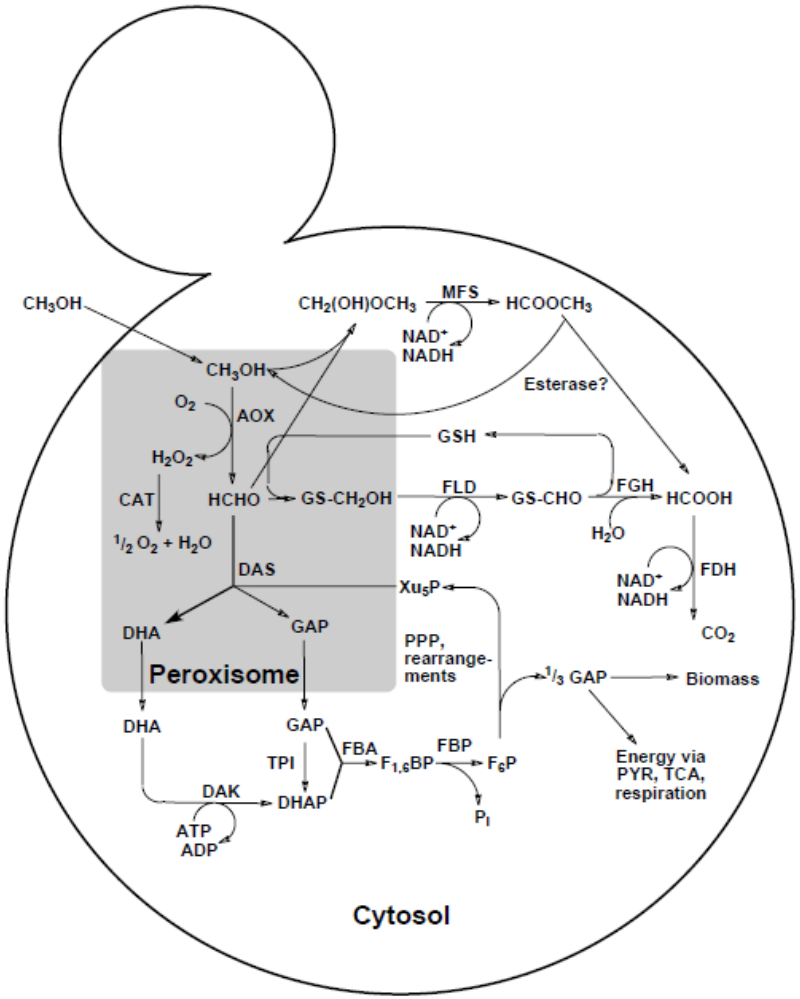


Figure 1.2: Methanol utilisation pathway in methylotrophic yeasts
The main pathways and the respective enzymes working in the methanol metabolism in methylotrophic yeasts are shown. AOX: alcohol oxidase, CAT: catalase, FLD: formaldehyde dehydrogenase, FGH: S-formylglutathione hydrolase, FDH: formate dehydrogenase, DAS: dihydroxyacetone synthase, TPI: triosephosphate isomerase, DAK: dihydroxyacetone kinase, FBA: fructose-1,6-bisphosphate aldolase, FBP: fructose-1,6-bisphosphatase, MFS: methylformate synthase; DHA: dihydroxyacetone, GAP: glyceraldehyde-3-phosphate, DHAP: dihydroxyacetone phosphate, $F_{1,6}BP$: fructose-1,6-bisphosphate, F_6P: fructose-6-phosphate, P_i: phosphate, Xu_5P: xylulose-5-phosphate, GSH: glutathione, PYR: pyruvate; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle (Hartner & Glieder, 2006).

1.3.1.2. Protein folding and post-transcriptional modifications

Methylotrophic yeasts do not hyper-mannosylate proteins as, for instance, *S. cerevisiae* do. As a result, recombinant proteins lack hyper-immunogenic terminal α-1,3-linked mannoses in N-linked glycans and the length of oligosaccharide chains is much shorter and much similar to those in mammalian cells. In this way the *Pichia pastoris'* Golgi and protein trafficking is much more similar to mammalian, plant and other higher eukaryote cells than *S. cerevisiae* and other yeasts (Figure 1.1). In fact, *P. pastoris* adds less than twenty mannoses per N-glycoside site. Thus, humanised methylotrophic yeasts offer potential for therapeutic proteins (Cregg *et al.*, 1993; De Schutter *et al.*, 2009; Hartner & Glieder, 2006; Heimo *et al.*, 1997; Wildt & Gerngross, 2005; Papanikou & Glick, 2009). More information regarding the in vivo synthesis of mammalian-like glycoproteins in *Pichia pastoris* can be found in Vervecken *et al.* (2004).

1.3.1.3. Strains

Many of the protocols aimed to engineer *Pichia pastoris* strains are derived from *S. cerevisiae*. Invitrogen Corporation (Carlsbad, CA, USA) offers a wide range of host strains (Table 1.2) and vectors, which can be used for research purposes but require an additional licence agreement for commercial uses. These kits make it relatively straightforward express recombinant proteins in *Pichia pastoris*.

Table 1.2: *Pichia pastoris* strains
List of the principal *Pichia pastoris* strains (Jahic *et al.*, 2006; Lin-Cereghino & Cregg, 2000).

Wild type strains	NRRL-Y-11430; X-33
Auxotrophic mutants defective in histidinol dehydrogenase	GS115; Y-11430; GS190; JC220; JC254; GS200; JC227; JC300-308.
Mutants defective in genes involved in methanol utilisation	KM71; MC 100-3
Protease-deficient strains	KM71; MC100-3; SMD1163; SMD1165; SMD1168; SMD1168 kex1::SUC2

Pichia pastoris GS115 (*his4*) is the most widely used Mut^+ strain. X-33 strains are isogenic to GS115, but they have been reverted from histidine auxotrophy (Cos *et al.*, 2005).

1.3.2. *Pichia pastoris* Growth

1.3.2.1. Methanol utilisation (Mut) phenotype

In *Pichia pastoris* glucose, ethanol, and glycerol repress both *AOX1* and *AOX2* promoters (Table 1.3). Even though both genes code for alcohol oxidase, *AOX1* expresses the vast majority of this enzyme. Therefore, by knocking out the *AOX1* gene, growth on methanol is slowed down drastically. This phenotype is called Mut^S (methanol utilisation slow). A knockout of *AOX2* will not decelerate growth on methanol and growth rates are comparable to the Mut^+ phenotype (wild type). However, by knocking out both genes, strains are unable to grow on methanol (Mut^-). Although there are some differences, this is true also for *Pichia methanolica*, in which both alcohol oxidase genes (*MOD1* and *MOD2*) act as in *Pichia pastoris* (Hartner & Glieder, 2006). Specific growth rates on methanol for Mut^+, Mut^S, and Mut^- phenotypes are 0.14, 0.04 and 0.00 h^{-1}, respectively (Cos *et al.*, 2006; Hartner & Glieder, 2006; Orman *et al.*, 2009). An uncompetitive inhibition growth model ($\mu = \mu_{max}S / (k_S + S + S^2 / K_I)$) shows that beyond a critical methanol concentration (S_{crit}) the theoretical maximum specific growth rate (μ'_{max}) declines from a maximum reached by increasing substrate concentration (Cos *et al.*, 2006; Stratton *et al.*, 1998).

Table 1.3: Effect of carbon sources on methylotrophic yeast genes
Methanol utilisation pathway genes in methylotrophic yeasts and repression/derepression effect of carbon sources on genes of methylotrophic yeasts (Hartner & Glieder, 2006).

Organism	Gene/ Promoter	Glucose, Ethanol	Glycerol	Methanol	Glycerol + methanol
Candida boidinii	*AOD1*	Repression	Derepr. (~3-30%)	Induction	Induction (~90%)
	FLD1	No activity	Derepression (~20%)	Induction	Induction (~70%)
	FDH1	Repression	No activity	Induction	Induction (~30%)
	CTA1	N/A	N/A	N/A	N/A
	DAS1	Repression	Derepression (~2%)	Induction	Induction (~70%)
Hansenula polymorpha (*Pichia angusta*)	*MOX*	Repression	Derepr. (~60-70%)	Induction	Induct. (~100%)
	FLD	N/A	N/A	N/A	N/A
	FMD	Repression	Derepression (~60%)	Induction	N/A
	DAS	N/A	N/A	N/A	N/A
	CAT	N/A	N/A	N/A	N/A
Pichia pastoris	*AOX1*	Repression	Repression	Induction	Repression
	AOX2	Repression	Repression	Induction	Repression
	FLD1	N/A	N/A	N/A	N/A
	ZZA1	N/A	N/A	N/A	N/A
Pichia methanolica (*Pichia pinus*)	*MOD1 (AUG1)*	Repression	Derepr. (~60-70%)	Induction	Induct. (~100%)
	MOD2 (AUG2)	Repression	No activity	Induction	Induct. (~100%)
	FLD	No activity	Derepression (~20%)	Induction	Induction (~70%)

N/A= Not affected

Given these three phenotypes, it is yet not clear which one is the most suitable for the expression of a given recombinant protein (Hellwig *et al*., 2001). However, it is stated that the Mut^S phenotype offers better results in terms of intracellular production, and both Mut^S and Mut^+ phenotypes can be used for extracellular production. As a consequence, it is suggested to test both systems. Furthermore, Mut^S is sometimes preferred as it is less sensitive to residual methanol in the medium. The process scale up is, therefore, simplified, since methanol levels in the culture can straightforwardly kept constant (Sreekrishna *et al*., 1997; Sreekrishna & Kropp, 1996; Stratton *et al*., 1998).

1.3.2.2. Auxotrophic host cells

HIS4 is a *Pichia pastoris* wild-type gene coding for histidinol dehydrogenase. *Pichia HIS4*-negative strains, also known as *his4*, are complemented with this gene to provide a selectable marker to isolate recombinant strains (Hollenberg & Gellissen, 1997).

Other possible marker genes commonly used in *Pichia pastoris* are *ADE1*, *ARG4*, *G418*, *URA3*, and Zeo^{r} (Lin-Cereghino & Cregg, 2000).

1.3.2.3. Growth Conditions

Temperature, pH, and medium composition have to be adjusted in order to reach the best conditions, which may be different among strains and/or foreign protein expressed (Cos *et al*., 2006). It has been shown that specific enzyme activities depend on the dilution rate of the methanol-limited chemostat. In *Pichia pastoris*, for instance, a reduction of the glycerol concentration in chemostat cultures increases the *AOX* activity. However, different microorganisms react differently to specific compounds and environmental conditions such as carbon source and dilution rate. As a result, it is suggested that genes involved in the Mut pathway may have individual specific regulation patterns. Thus, this diversity offers remarkable advantages to researchers during their strategy design, as they are able to optimise different parameters and strains to achieve the best outputs (van Dijken *et al*., 1976).

When grown on glucose or glucose/methanol media, some mutant strains of methylotrophic yeasts show high activities of Mut pathway enzymes. While wild type strains use a diauxic growth pattern, mutants are able to use glucose and methanol in parallel. It is suggested that a shift from methanol to glucose or ethanol triggers a micropexophagy or macropexophagy (autophagy of peroxisomes), respectively, in such mutants. In *Pichia pastoris*, when changed to ethanol-containing media, a cAMP pulse was found during the metabolic shift (degradation of *AOX* activity), indicating a

dependence on intracellular ATP levels for the mode of autophagy, triggered by the α-subunit of phosphofructokinase (Dunn *et al.*, 2005; Murray *et al.*, 1990).

1.3.3. *Pichia pastoris* recombinant protein expression

1.3.3.1. Regulation of *P. pastoris* regulators

In methylotrophic yeasts, promoters involved in the Mut pathway are strongly induced by methanol and repressed by ethanol and glucose (Table 1.3). After induction, alcohol oxidase (*AOX*), dihydroxyacetone synthase (*DAS*), and formate dehydrogenase (*FDH*) may reach titres up to 30, 20, and 20% of the total soluble proteins, respectively. However, in the nitrogen metabolism, genes involved in the dissimilation pathway are regulated in different ways. In fact, *FLD* and *FDH* can be induced by methylated nitrogen sources (e.g. methylamine and choline) and they are not repressed by glucose (Gancedo, 1998).

Promoters are not regulated in the same way in all methylotrophic yeasts, which have different number of genes coding for alcohol oxidase (the first enzyme in the Mut pathway). For instance, *Pichia pastoris* and *Pichia methanolica* control two different genes coding for such enzyme. These genes are *AOX1* and *AOX2*, and *MOD1* (*AUG1*) and *MOD2* (*AUG2*), respectively. Conversely, *Candida boidinii* (*AOD1*) and *Pichia angusta* (*MOX*) only code for one alcohol oxidase (Szamecz *et al.*, 2005). To date, there are no specific studies aimed to understand the role of *AOX2* in *Pichia pastoris*. However, in *Pichia methanolica* and in *Candida boidinii* it has been showed that the equivalent genes reduce the lag-phase during growth on 3% methanol. In fact, these alcohol

oxidases have different K_M values that help the microorganisms to adapt to different methanol concentrations in the media (Nakagawa *et al*., 2002).

P_{AOX1} and P_{AOX2}

P_{FLD1} and P_{AOX1} are the most transcriptionally efficient (transcription levels / theoretical maximum transcription levels) *P. pastoris* promoters, with AOX1 widely recognised as being the strongest (Duan *et al*., 2009). Methanol allows close control of AOX1 induction and glucose, glycerol, and other carbon sources tightly repress transcription (Figure 1.3). Both *AOX* operons in *Pichia pastoris* code for alcohol oxidase; however, 90% of the total *AOX* activity is performed by *AOX1* (Cregg, 1999).

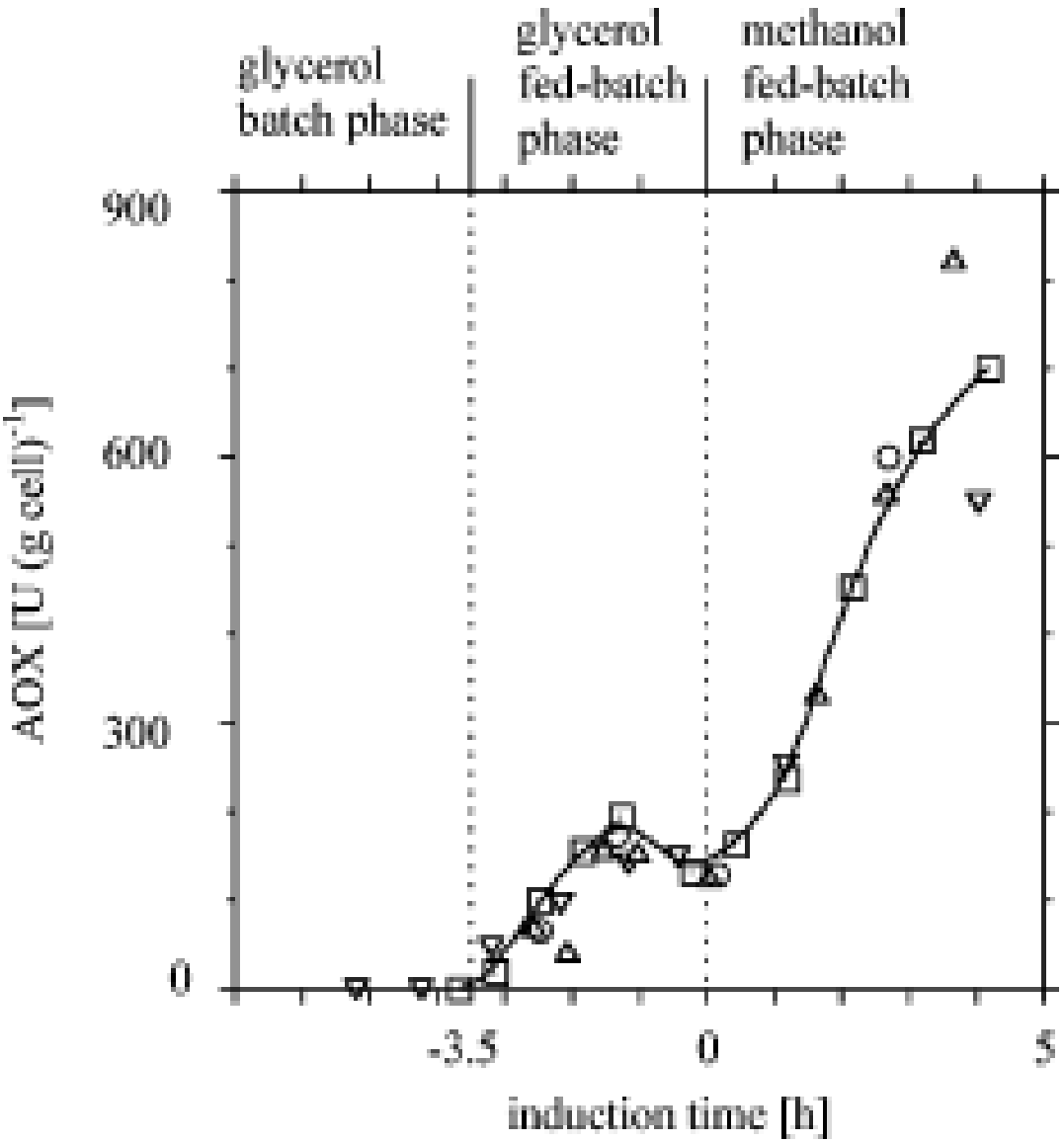


Figure 1.3: Alkaline phosphatase activity during the different fermentation phases
Specific *AOX* activity during the transition phase in four *Pichia pastoris* high-cell-density fed-batch cultivations (Jahic *et al.*, 2006).

1.3.3.2. *Pichia pastoris* Growth Conditions

Typically, fermentation protocols for the Mut^+ phenotype involve a biomass increase using a carbon source other than methanol (Pla *et al.*, 2006). There are several commercially available media for the production of *Pichia pastoris* high density biomass. Basal salt medium (BSM) is commonly used for preliminary growth studies. However, it generates some problems such as unbalanced composition, precipitates (over pH 5), and ionic strength. Other media aimed to reduce these problems have been formulated (D'Anjou and FM22), offering different ranges of basic elements (Table 1.4). Note that nitrogen is supplemented during the culture pH monitoring in BSM and FM22 media as NH_4OH. On the other hand, D'Anjou media supplements nitrogen in the initial formulation, and the pH is controlled by other means (Wegner, 1983).

Table 1.4: Media components comparison
Elemental content comparison between medium (BSM, FM22, D'Anjou) compositions (Cos *et al.*, 2006).

Element	BSM (g/L)	FM22 (g/L)	D'Anjou (g/L)
N	NH_4OH (pH control)	1.06 + NH_4OH (pH control)	4.24
P	12.27	9.76	2.73
K	11.05	18.74	3.45
Mg	1.47	1.15	0.46
Ca	0.27	0.12	0.10
S	5.51	5.46	5.47
Cl	--	--	0.17

The elements concentrations described in the table are the total amount of such element derived from all the used salts, so the value are not expressed in relation to the used salts. The listed elements are supplied by the following salts: Ammonium sufate, Phosphoric acid, Potassium sulfate, Potassium hydroxide, Potassium phosphate, Magnesium sulfate, Calcium sulfate, Manganese sulfate, Sulfuric acid, Ferrous sulfate, Cupric sulfate, Cobalt chloride, Zinc chloride.

Even though the influence of vitamins and micronutrients (Fe, Mn, Cu, Biotin) in these media has not yet been widely studied, it has been shown that production can be increased by adding vitamins and trace salts in BSM media. Aeration and agitation cause foam formation in culture media. Thus, addition of antifoam (silicone based) agents is highly recommended. However, they may affect oxygen transfer rate and cell growth rate (Boze *et al.*, 2001; Kock *et al.*, 1995).

1.3.3.3. Methanol Feed strategies for Transgene Induction

Two or three activation steps are usually required for methanol-induced gene expression. They are gene activation independent of methanol (glycerol or glucose derepression done by adding the carbon source at growth-limiting rates) and methanol-specific induction (Egli *et al.*, 1980). For the Mut^S and Mut^- phenotypes, mixed feeds (methanol and another carbon source) are recommended (required for Mut^-) to induce protein expression (Guarna *et al.*, 1997; Zhang *et al.*, 2003). However, mixed feed fermentations may increase the overall production level, even though the partial repression of *AOX1* by residual glycerol does not allow reaching maximal levels of proteins. Sometimes, a transition phase before methanol-only feed helps to consume residual glycerol and by-products (e.g. ethanol) generated during batch phase (levels should be low as non-fermentable carbon sources are usually employed) by a short starvation period (Inan & Meagher, 2001).

After induction with methanol, *Pichia pastoris* continues to grow on methanol to high-cell-density also in oxygen-limiting conditions. In fact, when oxygen becomes a limiting parameter, *P. pastoris*, which is an obligate aerobe, does not switch its

metabolism as other microorganisms (e.g. *S. cerevisiae*) do (Charoenrat *et al.*, 2005; Trentmann *et al.*, 2004). Shake-flask cultures of methanol-induced *Pichia pastoris* reach production levels of about 5% of the total soluble proteins. On the other hand, values over 30% can be reached in fermenter by growth-limiting cultures in methanol (Burrowes *et al.*, 2005) Those high methanol feed-rates are only possible if methanol is supplied at limiting rate and therefore consumed immediately by the cells. If those methanol feed rates are feeded to the cells at non-limiting conditions, methanol will accumulate in the vessel becoming toxic to the cells. As a result, a good methanol monitoring system is very important. For instance, mid-IR spectroscopy allows to detect methanol levels on-line by measuring it directly from the broth at a wavelength of 1018 cm^{-1} (Schenk *et al.*, 2007).

Methanol influences both cell physiology and protein production rate, as it is utilised simultaneously as carbon source and gene inducer. Thus, maintaining steady methanol levels is a key parameter for reproducibility studies. Because of its double role, this is a difficult step. In fact, exponential growth, which also increases production rate, increases methanol consumption in a nonlinear manner (Pla *et al.*, 2006). Furthermore, methanol levels influence productivity in two different ways. First, high levels of methanol (over 3-5 g/L) are toxic to the cells, leading to accumulation of formaldehyde and hydrogen peroxide, and consequently death of the cell (Zhang et al., 2000). On the other hand, low levels of methanol trigger proteolytic degradation of heterologous proteins, which results in lower productivity. Therefore, methanol concentrations up to 3 g/L maximise specific production rates and growth rates. However, the concentration of

methanol can be considerably reduced by using fed-batch methods (Pla *et al.*, 2006; Orman *et al*., 2009).

Pla *et al.* (2006) showed that the Mut^+ phenotype produces up to 2 g/L of scFv antibody at a concentration of methanol of 0.1 g/L, where the maximal accumulated methanol concentration was 1.0 g/L. On the other hand, low methanol concentrations produce almost no effects on the growth of Mut^S strains and no inhibition was observed at a concentration of 5.0 g/L of methanol. In addition, they observed concentrations of about 6 g/L of scFv antibody when cells where grown at a concentration of 0.1 g/L of methanol. The dry cell weight under these conditions was approximately 400 g/L. Thus, production of extracellular heterologous proteins in *Pichia pastoris* is increased with lower concentrations of methanol. It has also been proven that the Mut^S phenotype produces higher protein yield, whereas the Mut^+ phenotype offers higher specific production rate (Pla *et al*., 2006).

1.3.3.4. Sorbitol as Supplementary Carbon Source

Sorbitol does not induce or repress AOX promoters and as such it is stated that using sorbitol instead of glycerol in mixed substrate methods reduces cell growth rate, but increases specific product formation rates. The use of mixed substrates further increases productivity and cell density, and reduces induction time (Orman *et al*., 2009). Compared to methanol-only feeds, a quantitative analysis of mixed feeds of sorbitol and methanol for the production of recombinant proteins with *Pichia pastoris* showed that 1.3-fold increase in productivity and a 38% reduction in generated heat and oxygen consumption

may be achieved by using a 43% C-mol C-mol^{-1} fraction of methanol in the mixed sorbitol/methanol feed medium. Compared to glycerol, sorbitol generates lower heat as its enthalpy of combustion is lower than glycerol (-549.5 kJ C-mol^{-1}), methanol (-727 kJ C-mol^{-1}), and mannitol (-507.8 kJ C-mol^{-1}, very close to sorbitol). This physical characteristic of sorbitol not only reduces generated heat, but also facilitates oxygen transfer rates and reactor cooling, which are two main technical issues for high cell density techniques. In addition, control of residual sorbitol concentration is less critical than mixed feeds of methanol and glycerol, since it is a non-repressive substrate and, therefore, sorbitol accumulation does not affect proteins' production (Jungo *et al.*, 2007).

1.3.3.5. Direct Methanol Detection Methods

Classical off-line methods for methanol monitoring are gas chromatography, HPLC, enzymatic reactions, and NIRS (near infrared refractance spectroscopy). Though, on-line monitoring is usually more efficient. Actually, some off-line techniques can be automated and become on-line as, for example, the sequential injection analysis (SIA). The drawback is that these methods still require samples, so contamination probability is increased and volume variations can be significant (Cos *et al.*, 2006). Methanol levels are frequently controlled by dissolved oxygen "spiking" ("DO spiking"). In fact, oxygen levels vary accordingly to methanol levels and drastic methanol concentration shifts are generated, by making this method unattractive for industrial applications. Ideally, methanol should be added in continuous instead of manual pulses to reduce fluctuations in dissolved oxygen and reduce starvation times. Another approach solved the problem

by targeting constant dissolved oxygen levels and oxidative capacity through a metabolic feed controller scheme. However, this approach is not suitable for mixed feed fermentations as dissolved oxygen is the only tracked parameter (Ruottinen *et al.*, 2008). In addition, increased DO leads to increased methanol feed rate, and when critical levels are reached, subsequent methanol addition will increase its accumulation and toxicity (Cos *et al.*, 2006).

Alternatively, on-line on/off methanol controllers may be employed. Unfortunately, these monitoring sensors are only suitable for shake flasks cultures, since in large bioreactors methanol levels may considerably fluctuate. In fact, as the culture scale increases, mixing becomes inadequate and areas of higher/lower methanol concentration are found within the used vessel (Damasceno *et al.*, 2004). Alternatively, other on-line methods (e.g. silicone tubing methanol sensor) during large-scale cultivation may help to control toxic methanol concentrations and avoid safety hazards. Similarly, gas analysers simultaneously measure levels of carbon dioxide and oxygen. However, they have low sensitivity, so methanol concentration monitoring is not efficiently carried out in methanol-limited processes (Hong *et al.*, 2002; Jahic *et al.*, 2003; Wagner *et al.*, 1997).

1.3.3.6. Indirect Methanol Detection Methods

Methanol accumulation can also be indirectly measured by a DOT (dissolved oxygen tension) response generated through a methanol feed pause. By repeating this process several times, the minimal DOT level is achieved (usually 20% air saturation)

and the methanol feed rate is then kept constant. Feed-back control loops can be employed to keep DOT constant (Teng & Samyudia, 2011). In continuous and semi-continuous cultures, Proportional-Integral-Derivative (PID) control may offer higher level of control over Mut^+ phenotypes. An improved version, closed-loop PID control, offers the opportunity to regulate the methanol induction levels for all Mut phenotypes of *Pichia pastoris* and it does not require prior knowledge on culture growth parameters. This system also allows for comparison studies between Mut^+ and Mut^S phenotypes for the production of heterologous proteins. In fact, this system allows cells to grow 35% faster than with on/off systems (Pla *et al.*, 2006).

1.3.3.7. Bioreactor culture techniques

Standard methanol-limited fed-batch (MLFB)

This system has been designed as a four-stage control process, which is aimed to control the repression/derepression and induction of *AOX1* as well as the oxygen consumption at high-cell-densities. The first phase is a batch growth on Glycerol. This phase is aimed to rapidly produce high amounts of biomass on Glycerol-containing media, and is ended at Glycerol exhaustion (DO increases sharply). Standard protocols suggest performing this step using a glycerol concentration of 40 g/L as higher concentrations inhibit growth and extended glycerol feed seems to breakdown the production of recombinant proteins. During this phase alcohol oxidase is not produced by cells and, therefore, a sudden increase of methanol in the media will lead to cell death. This problem is overcome by a short fed-batch growth on glycerol-limited medium (stage two

– transition phase). This stage allows for derepression of the *AOX1* promoter and a consequent higher initial methanol feed rate. During this stage, growth rate slows down from 0.26 h^{-1} to 0.18 h^{-1} (SMD1168 strain), which is sufficient for *AOX1* derepression and for high biomass density production (up to 30 g/L).

Sometimes, the transition phase is complemented with low amounts of methanol (under 4 g/L), aimed to ease and speed up the *AOX1* promoter derepression and induction. This strategy is called mixed feeding and it is performed at limited glycerol feeding rate. In the standard strategy, exponential glycerol feed rate (Figure 1.4) is usually preferred to the constant one as it offers higher biomass productivity and constant growth rate, where the maximum glycerol specific consumption rate is 0.0688 g g^{-1} h^{-1} (Cos *et al.*, 2006; Zhang *et al.*, 2006)).

$$F(t) = \frac{\mu X_0 V_0}{G_i Y_{X/G}} e^{\mu t}$$

Figure 1.4: Equation: exponential glycerol feed profile
This equation estimates the exponential glycerol feed profile (Jahic *et al.*, 2006)
$F(t)$ (L/h) is the glycerol feed rate at time t(h); μ (h^{-1}) is the desired specific growth rate; X_0 (g/L) is the initial biomass concentration; V_0 (L) is the initial volume; G_i (g/L) is the glycerol concentration in the feed; $Y_{X/G}$ (g/g) is the biomass per glycerol yield coefficient.

The following stage is the methanol induction. This step is critical as oxygen consumption rate and alcohol oxidase activity increase rapidly. However, just before induction its activity is very low. Thus, methanol may easily accumulate and creating threats to cells survival and *AOX* activity. A good methanol monitoring can be achieved either by direct or indirect analysis as described above. After induction, the methanol concentration drops rapidly to levels that are difficult to measure. At the same time, cells continue to grow at decreasing rate. Therefore, the final biomass is proportional to the methanol feed rate, which is influenced by the oxygen transfer rate. All in all, by increasing pure oxygen concentration and the total air pressure, higher methanol feed rates are possible, leading to higher productivity of biomass and heterologous proteins during the methanol-limited fed-batch stage (Jahic *et al.*, 2006).

As stated above, *Pichia pastoris* is able to produce proteins up to g/l levels. For instance, a volumetric yield of 4 g/l of a humanised single-chain variable domain fragment antibody (A33scFv) was expressed by *Pichia pastoris* (Mut^S) at standard methanol feed rate (0.1% v/v) and pH (pH 6.0). Damasceno *et al.* (2004) showed that an higher methanol concentration (0.5% v/v) increases protein production to 4.3 g/l after 72 hours incubation. Protein yield was also affected by pH, reaching a volumetric yield of 4.88 g/l at lower pH (pH 3.0). In terms of biomass accumulation, cells were not affected by pH variation, but they responded positively to higher methanol concentrations (Damasceno *et al.*, 2004). Similarly, it was showed that a 50% faster methanol feed rate (coupled with oxygen enrichment to 34% O_2) during the methanol induction phase resulted in 14% higher biomass accumulation (*Pichia pastoris* Mut^+), corresponding to an OD_{600} of about 580 [-] (Jahic *et al.*, 2002). In this case, specific productivity did increase

proportionally to the methanol feed rate. However, a further increase of the methanol feed rate methanol induction augmented biomass accumulation by 21%. On the other hand, the increased methanol feed rate did not result in any augmented specific product formation rate (Jahic et al., 2002).

Even though *Pichia pastoris* secretes low levels of endogenous proteins, the proteolytic activity in high cell density fermentations increases. The final product can be mainly degraded by intracellular proteases released after cell lysis/death. Vacuolar proteases are the principal source of protein degradation in the medium as they have low substrate specificity and they do not require ATP. Among these proteases it is possible to find endoproteinase A and B, carboxypeptidase Y and S, aminopeptidase I and yscCo. Therefore, high production levels tend only to be achieved if high-cell viability is maintained throughout the process. To overcome this problem engineered strains of *Pichia pastoris* have been created. Genes *PEP4* (encodes protease A) and *PRB1* (encodes protease B) have been inactivated in these mutants leading to a substantial reduction of protease activities. Interestingly, in these mutants (*pep4* and *pep4*/*prb1*) it has been shown also a reduction in carboxypeptidase Y activity. Note that in the *prb1* mutant only protease B activity has been eliminated (De Schutter *et al.*, 2009).

Regular western blot analyses may offer a starting point in the evaluation of the proteolytic activity against the target protein. Eventually, the culture medium can be supplemented with protease inhibitors such as PMSF (for serine proteases). In addition, pH can be adjusted to reduce protease activity as the *Pichia pastoris* pH range (optimum between 5 and 6) is relatively large (from pH 3 to pH 7) and it does not influence the growth rate. Alternatively, it is suggested that media enriched with amino acid rich

supplements (e.g. peptone and casamino acids) reduce product degradation since alternative and competing substrates are available for proteases. The drawback, however, is that these supplements are not suitable for large scale production because of their cost and the negative effect they have on downstream process when it comes to their removal. Finally, later it will be explained that temperature-limited fed-batch techniques (TLFB) may also reduce proteolytic activity (Cos *et al.*, 2006).

Continuous techniques

Higher volumetric productivity, product quality, and uniformity as well as lower proteolytic activity, protein oxidation, and protein inactivation can be achieved by continuous cultivations. However, this operational mode is usually limited to the Mut^+ phenotype as the Mut^S phenotype is limited by its low maximum specific growth rate that make it very difficult for these strains to cope with the operational dilution rates (usually between 0.005 h^{-1} and 0.09 h^{-1}). In fact, the highest production rate is achieved at the highest dilution rate (0.09 h^{-1}). On the other hand, the Mut^S phenotype can reach high production rates when grown on mixed substrates at low dilution rates (between 0.005 h^{-1} and 0.035h^{-1}). Though, productivity is maximised at intermediate dilution rates. Different continuous production strategies are reported in Table 1.5 (Boze *et al.*, 2001; Curvers *et al.*, 2001).

Table 1.5 : Continuous cultures comparison

Protein	Promoter	Operation	Mixed substrates	D	Protein	Productivity	$Y_{P/X}$	Specif productivity
				h^{-1}	mg/L	mg/L h	mg prot/g X	mg prot/gX h
Porcine FSH	*AOX* Mut^S	Fed-Batch	Methanol	----	45	0.3	1.2	0.014
			Gly-met	----	27	0.2	0.6	0.011
			Sor-met	----	93	0.6	0.9	0.016
			Sor-met-cas	----	72	0.5	1.4	0.017
			Sor-met-ye	----	113	0.8	1.5	0.019
			Sor-met-v-ts	----	187	1.3	2.5	0.035
		Continuous	Methanol	0.012	170	2.0	2.8	0.033
			Met-sor-v-ts	0.005	282	1.4	3.6	0.018
				0.010	121	1.1	1.8	0.018
Sea raven AFP	*AOX* Mut^S	Fed-Batch	Gly-met	μ = 0.03	180	2.3	3.0	0.038
				μ = 0.07	120	2.4	2.0	0.040
		Continuous		0.01	60	0.6	1.5	0.015
				0.02	40	0.8	1.0	0.020
				0.03	50	1.5	1.3	0.040
				0.05	15	0.8	0.5	0.025
				0.07	15	1.1	0.7	0.050
				0.08	15	1.2	0.8	0.060
				0.09	15	1.4	0.7	0.065
Bovine lisozyme	*AOX* Mut^S	Fed-batch	Methanol	----	250	1.2	5.2	0.026
			Gly:met 4:1	----	180	3.4	2.3	0.046
			Gly:met 2:1	----	290	4.8	3.7	0.062
			Non-limiting methanol	----	375	5.6	4.0	0.093
	AOX Mut^+	Fed-Batch	Methanol	----	450	7.7	5.6	0.154
		Continuous		0.035	600	12-15	----	----
Sea raven AFP	*AOX* Mut^+	Fed-Batch	Methanol	----	475	5.3	3.2	0.035
		Continuous		0.038	368	14	----	----
				0.062	339	21	----	----
				0.072	347	25	4.3	0.31
Ovine interferon-δ	*AOX* Mut^+	Continuous	Methanol	0.008	45	0.4	0.9	0.007
				0.0136	149	2.0	2.3	0.031
				0.035	85	3.0	1.3	0.047
				0.05	39	2.0	0.5	0.027
h-chitinase	*GAP*	Fed-Batch	Glucose	----	300	2.5	7.5	0.063
				----	450	2.7	6.8	0.040
		Continuous		----	300	15	2.1	0.150
				0.042	250	6.1	2.8	0.067

Gly = glycerol; met = methanol; sor = sorbitol; cas = casamino acids; ye = yeast extract; v = vitamins; ts = trace salts (Cos *et al.*, 2006).

1.3.3.8. Alternative culture techniques

Shake flasks cultivations

In shake flasks cultivations methanol is added during the late growth on glycerol, allowing cells to switch their metabolism earlier and starting a batch culture on methanol, which is the only source of carbon used during the whole bioreactor process. This method allow for biomass production up to 13.3 g/L (Ramchuran *et al.*, 2005).

Mixed-feed technique

Another method is aimed to reduce the transition phase. In fact, concomitant derepression and induction of the AOX1 promoter reduces this phase to two hours in a process where methanol adaptation happens during the glycerol-limited feed, which usually take place after one hour of culture on glycerol only. This is done by adding 1 to 4 g/L (optimum: 1.5 g/L) of methanol while the glycerol feed rate is linearly reduced. Thus, alcohol oxidase synthesis is strongly promoted as a result of simultaneous *AOX1* derepression and induction. This technique has been shown to be able to increase productivity of *Rhizopus oryzae* lipase by 13.6-folds (Balamurugan et al., 2006). In contrast to the wild type (Mut^+), Mut^S strains are usually grown on mixed methanol and glycerol feed, as these strains exhibit longer induction times (as they mainly rely on the *AOX2* gene, so their growth on methanol is reduced (Balamurugan et al., 2006). In addition, methanol has a high heat of combustion (-727 kJ/C-mol) with generates extensive heat in fed-batch stages. On the other hand, glycerol produces less enthalpy of combustion (-549.5 kJ/C-mol). As a consequence, mixed feeds generate lower heat compared to methanol alone and, therefore, oxygen consumption is also reduced as glycerol oxidation requires lower amounts of oxygen. Thus, as oxygen transfer causes problems in high cell density cultures, any technique that reduces its consumption would be advantageous as long as it does not affect productivity levels (Jungo *et al*., 2007).

In mixed substrate cultures the methanol concentration is lower than methanol only cultures, and the *AOX* activity is usually found to be higher. Since methanol oxidation causes growth limitations, substrate oxidation in the cell in maintained by increasing the production of the rate-limiting enzymes which, in turn, increase

productivity levels. This phenomenon is also seen in chemostat cultures (methanol only) when dilution rate is decreased. The effect of methanol on protein production using the mixed-feed technique has been described by Jungo *et al.* (2007). They used a linear increasing gradient between 0.5 and 1.0 C-mol/C-mol of methanol in the feed medium of a steady state continuous culture of *Pichia pastoris* (Mut^+). The oxygen consumption, carbon dioxide production rate, and the heat generation rate all increased when the methanol concentration was increased. In addition, they showed that low levels of glycerol do not repress the *AOX1* promoter, and that *AOX* activity increases by 1.6 fold in 0.5 C-mol/C-mol of methanol fraction compared to methanol as a sole source of carbon. Higher volumetric productivity (1.1 fold) is achieved with a methanol fraction of 62%, which also significantly reduce the oxygen consumption rate and the heat generation. Therefore, mixed feeds of glycerol (40%) and methanol (60%) at 0.06 h^{-1} specific growth rate during the induction phase are preferred to the standard methods using methanol only (Jungo *et al*., 2007).

Temperature-limited fed-batch

With the objective of avoiding oxygen limitation at high cell density, a temperature limitation (TLFB technique) replaces the methanol limitation. This technique is somewhat similar to MLFB processes, where the temperature-limited fed-batch process is initiated when DOT reaches 25% air saturation, which is the set point for the temperature controller (Jahic *et al*., 2003). Optimum production temperature in *Pichia pastoris* is 30°C and above 32°C production is strongly impaired. However, it has been shown that production of heterologous proteins can be increased by reducing the working

temperature (Li *et al*., 2001). With a constant concentration of methanol (0.3 g/L), in the TFLB technique the temperature is increased or reduced when the DOT is above or below 25%, respectively. In other words, by keeping the DOT constant throughout the process, oxygen limitation is avoided. However, large bioreactors are usually not well suited to control temperatures below 12°C. As a result, oxygen limitation at these temperatures is avoided by switching to constant temperature and limited methanol feed, which are adjusted to keep the DOT constant. In bioreactors larger than 20 L, the final temperature might be higher than 12°C. In both cases, while growth rates are comparable to the MLFB technique, low temperatures allow for reduced protease activities, reduced cell dead, higher productivity, and higher cell density (Wu *et al*., 2012). Similarly, lower temperature allows for more correctly folded proteins as folding stress is reduced. In addition, cells adapt themselves better to methanol, and limitations in oxygen and methanol are alleviated (Zhao *et al.*, 2008; Gasser *et al*., 2007).

Oxygen vectors

To avoid oxygen limitations, oxygen vectors can be employed. These are hydrophobic liquids (e.g. *n*-hexane, *n*-heptane, *n*-dodecane) in which oxygen has higher solubility than in water. Concentrations used are between 0.5 and 2% and the main advantages are, in addition to an increased oxygen transfer rate, low costs, and low possibility of danger (Zhang *et al.*, 2008).

Oxygen-limited fed-batch

As *Pichia pastoris* is an obligate aerobic organism on methanol media and as it do not produces secondary fermentative products, oxygen-limiting (OLFB) techniques can be employed. In addition, by working at DOT $\approx$ 0, the oxygen transfer rate is increased just as the methanol consumption is. Similarly, high-pressure processes (MLFB process run at 1.9 bars total pressure instead of 1.2 bars) have also been shown to increase methanol and oxygen consumptions. In the OLFB technique, the methanol feed is kept constant at 0.35 g/L, resulting in 35% higher oxygen consumption (Charoenrat *et al.*, 2005). Higher biomass productivity and cell viability have also been reported. However, even if higher productivity was recorded, secreted proteins were found to be less homogeneous than in MLFB processes (Charoenrat *et al.*, 2006).

Increased methanol feed

Finally, Khatri (2011) described in his work a biomass increase after methanol induction when higher methanol feeds are used (up to 4%). 0.3% (v/v) methanol was compared with 1 and 3%. It is also stated that higher methanol supply can reduce oxygen uptake rate and therefore offering higher product quality and quantity. Similarly, Jahic *et al.* (2002) found that a methanol feed increased by 50% increases biomass formation. Induction time-point will also play an important role on the amount of methanol consumption. In fact, earlier induction (shorter glycerol phase – two hours) will increase oxygen demand and product accumulation is delayed. Feeding glycerol at an exponential rate for 18 hours resulted in immediate protein release after induction. However, product final concentrations were similar in all cultivations (2h, 12h, and 18h glycerol phases). In

this work glycerol was fed for about 8 hours at a constant rate, and therefore some delays were expected to observe some protein accumulation after induction (Khatri, 2011).

1.3.4. *Pichia pastoris* Genetic Modification

1.3.4.1. Genome integration and stability

Expression vector integration into the *Pichia pastoris* genome maximise the stability of recombinant proteins (Cregg *et al*., 1993). Linearised vectors that share sequences (AOX1 locus or his4 locus) with the host genome integrate via homologous recombination into either the *AOX1* locus or the *HIS4* locus. In absence of selective pressure these mutants show high stability independently from the number of integrated copies (Hollenberg & Gellissen, 1997). For instance, the vector pPIC9K from Invitrogen Corporation offers the possibility to detect multiple-copy of the foreign gene through increasing selective pressure. In fact, a single copy of the integrated vector confers resistance to Geneticin® up to 0.5 mg/ml. However, multiple integrations (7-12 copies) offer the cell a stronger resistance capability (up to 4 mg/ml of antibiotic) called drug hyper-resistance. The vector copy number can also be accurately quantified by dot-blot hybridisation. The strategy for a multiple expression cassette integration is shown in Figure 1.5. Even though an increased gene copy number usually led to increased production of intracellular foreign proteins, extracellular products may be impaired by a maximised gene copy-number (Cos *et al.*, 2005).

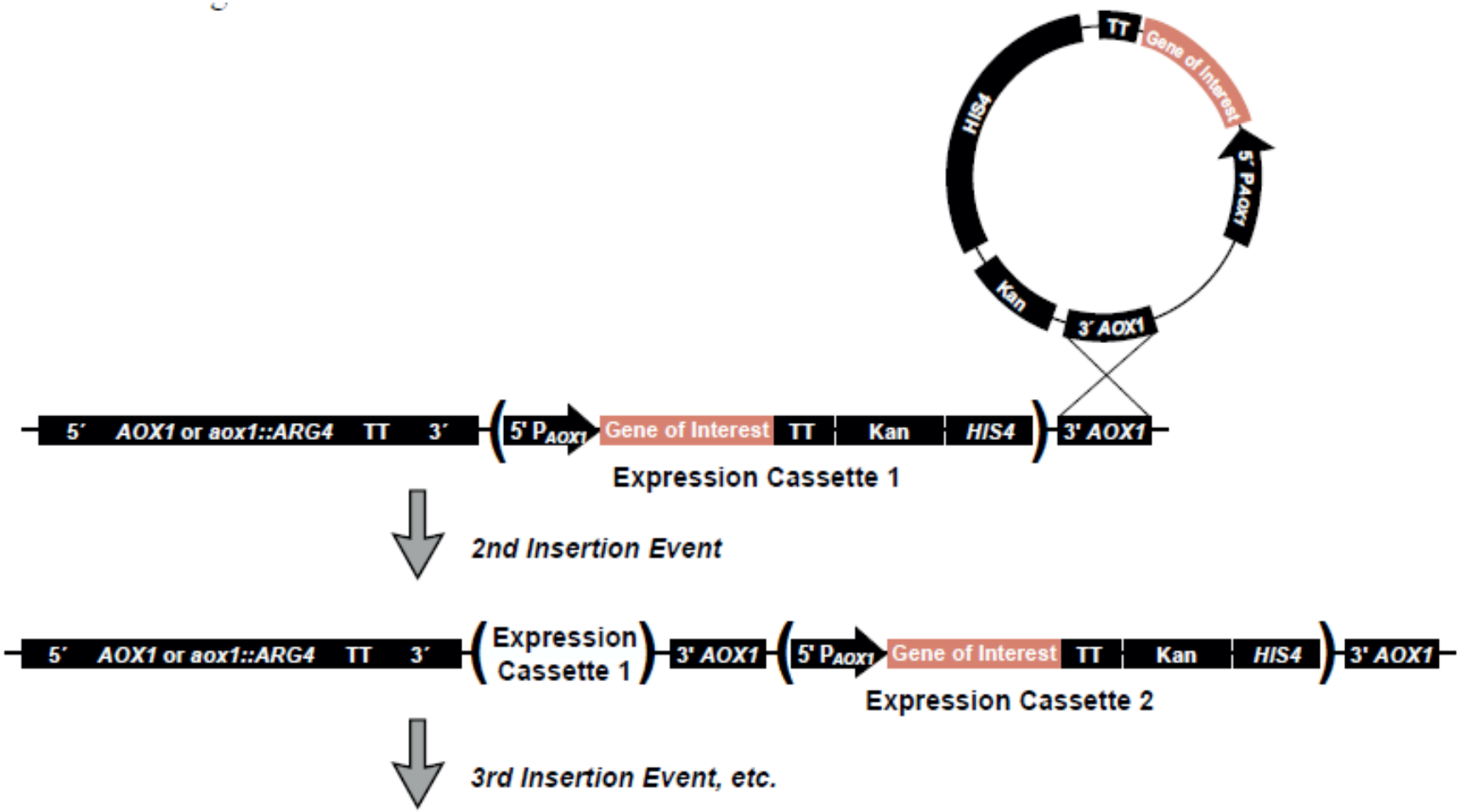


Figure 1.5: Expression cassette multiple insert
Multiple insertion of the expression cassette linked to the Kanamycin gene (http://www.invitrogen.com).

In serial cultures without methanol induction *Pichia pastoris* clones are able to retain their original vector copy-number for 35 generations. Genetic stability can strongly influence production yields as the loss of the foreign gene will trigger a competitive growth advantage over stable clones during the fermentation process. Integrating vectors (see above) are increasingly excised as their copy-number increases, since the likelihood of excisional recombination increases in longer tandem array of vectors integrated. In other words, genetic stability decreases with increased copy-number. The plasmid instability is due to two main factors: structural and segregational instabilities. The former is caused by deletion, insertion, or recombination and the latter by an uneven partitioning of plasmids during cell division. In *Pichia pastoris*, segregational instability is overcome by chromosomal integration. However, in some cases a loss of the foreign gene after a number of generations it has been reported, indicating that structural stability in P*ichia pastoris* still needs to be optimised as methanol induction increases the possibility of excisional recombinant events. However, even in presence of methanol, low copy-number clones have been shown to have higher stability (Zhu *et al.*, 2009).

1.3.4.2. Recombinant protein expression vectors

pAO815 (Figure 1.6) from Invitrogen Corporation is a typical *Pichia pastoris* expression vector. Its main features are the *AOX1* promoter and transcriptional terminator, the *Pichia pastoris* histidinol dehydrogenase gene for *his4* hosts selection, the *E. coli* pBR322 origin of replication and ampicillin resistance, and a unique restriction site (*Eco*RI) for insertion of heterologous proteins. The 3' *AOX1* sequence is aimed to target plasmid integration at the *AOX1* gene locus, helped by a linearization of the plasmid with

*Sal*I or *Stu*I (insertion at *HIS4* – generates *His*$^+$ Mut$^+$ in *Pichia pastoris* GS115 and *His*$^+$ Mut^S in *Pichia pastoris* KM71) or with *Bgl*II (gene replacement at *AOX1* – generates *His*$^+$ Mut^S in *Pichia pastoris* GS115), which stimulate single-crossover type integration events (Cregg et al., 1993).

Unlike pAO815, pPIC9K (Figure 1.7) carries a signal sequence to target proteins into the *Pichia pastoris* secretory pathway (http://www.invitrogen.com). This signal is a 269 bp α-Factor secretion signal fragment, also known as α-MF (mating factor) prepro from *S. cerevisiae* (Cregg *et al.*, 1993). This mating factor induces the Sec61p-mediated translocation of proteins into the endoplasmic reticulum (De Schutter *et al.*, 2009). Secretion of this factor and its associated protein is a complex process, which require sequence events ranging from correct transport between the endoplasmic reticulum (ER) and the Golgi to specific proteases cleavage of the secretory signal (Nallaseth & Anderson, 2013). The pPIC3.5K vector from Invitrogen Corporation is similar to the pPIC9K vector, but it does not carry the secretion signal fragment for the secretion of heterologous proteins into the medium. For single copy integrations, these plasmids are available as pPIC9 and pPIC3.5 (Figure 1.7), which does not carry the Kanamycin gene (http://www.invitrogen.com). The vectors pPICZ and pPICZαA,B,C are similar to the ones described above, but they carry the gene coding for Zeocin resistance. Zeocin multiple copy number are screened by increased concentrations of the drug, and selection is usually done in YPDS plates (3-4 days): 1% yeast extract, 2% peptone, 2% glucose, 1M sorbitol, and 2% agar (Cos *et al*., 2005; Guo *et al*., 2009). Similarly, pGAPZ and pGAPZαA,B,C vectors offer the same features, but the foreign protein is put under the control of the *GAP* promoter instead of the *AOX1* promoter (http://www.invitrogen.com).

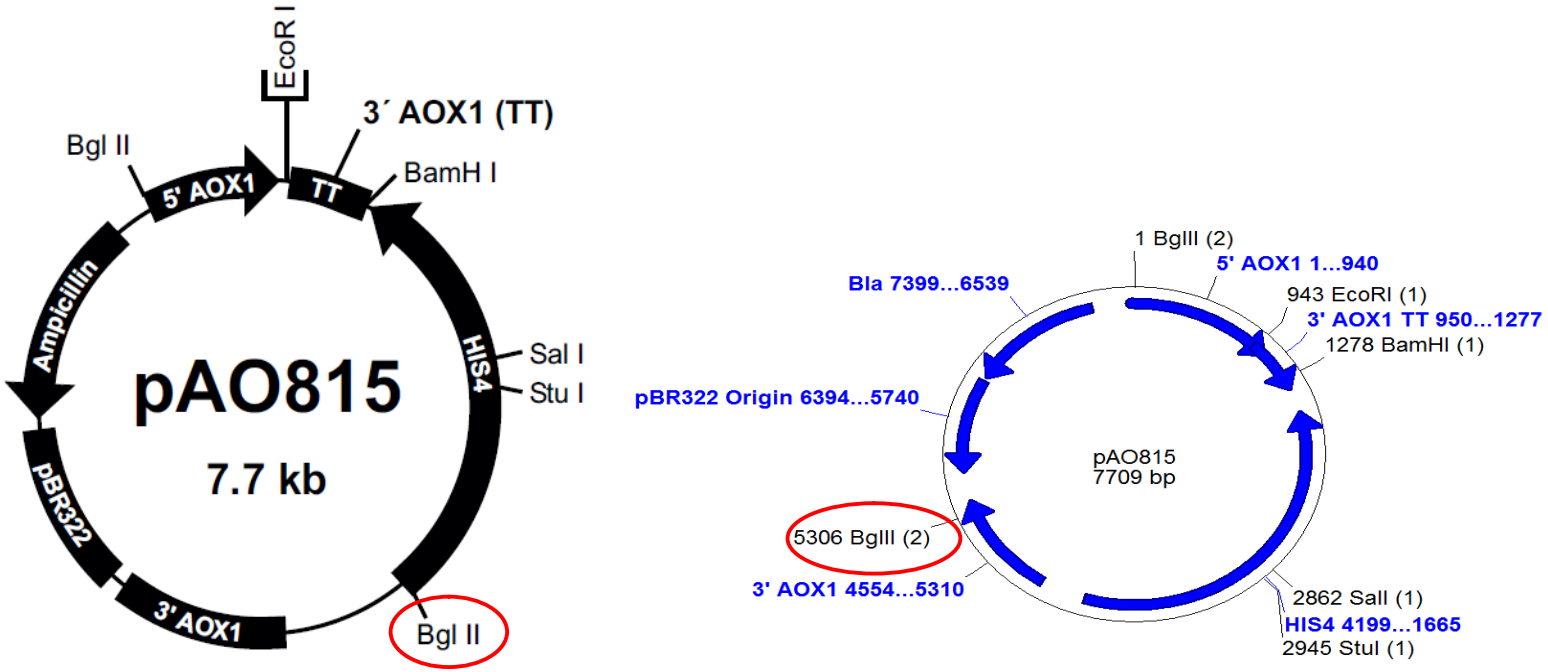


Figure 1.6: pAO815 vector map

pAO815: 7709 nucleotides. 5' *AOX1* promoter fragment (1-940); 5' *AOX1* primer site (855-875); *Eco*RI site (943-948); 3' *AOX1* primer site (1024-1044); 3' *AOX1* transcription termination TT (950-1277); *HIS4* ORF (4199-1665); 3' *AOX1* fragment (4554-5310); pBR322 origin (6394-5740); Ampicillin resistance gene (7399-6539) (http://www.invitrogen.com). Note that one of the two *Bgl*II sites is not on the *HIS4* region, but it is at position 5306 (3' AOX1 region).

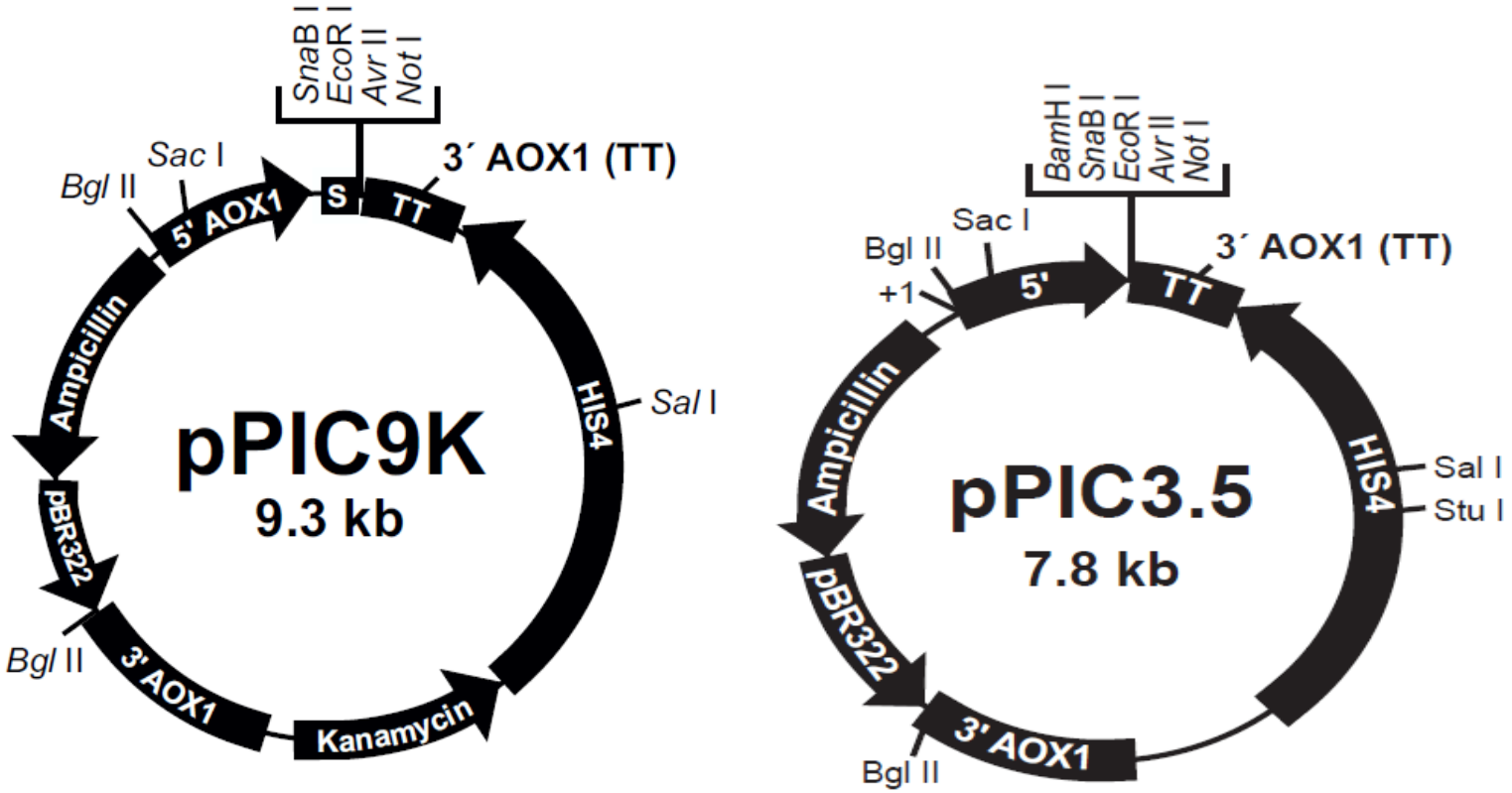


Figure 1.7: pPIC9K and pPIC3.5 vectors maps
pPIC9K: 9276 nucleotides. 5' *AOX1* promoter fragment (1-948); 5' *AOX1* primer site (855-875); *α*-Factor secretion signal(s) (949-1218); *α*-Factor primer site (1152-1172); Multiple Cloning Site (1192-1241); 3' *AOX1* primer site (1327-1347); 3' *AOX1* transcription termination TT (1253-1586); *HIS4* ORF (4514-1980); Kanamycin resistance gene (5743-4928); 3' *AOX1* fragment (6122-6879); pBR322 origin (7961-7288); Ampicillin resistance gene (8966-8106). **pPIC3.5:** 7751 nucleotides. 5' *AOX1* promoter fragment (1-937); 5' *AOX1* primer site (855-875); Multiple Cloning Site (938-968); 3' *AOX1* primer site (1055-1075); 3' *AOX1* transcription termination TT (981-1314); *HIS4* ORF (4242-1708); 3' *AOX1* fragment (4598-5354); pBR322 origin (6436-5764); Ampicillin resistance gene (7442-6582) (http://www.invitrogen.com).

1.3.4.3. *Pichia pastoris* genetic modification

Vectors can be inserted into the *Pichia pastoris* through three main methods: Electroporation, spheroplast generation, or whole cell method such as by lithium chloride or polyethylene $glycol_{1000}$. Just as *S. Cerevisiae*, *Pichia pastoris* easily accept foreign DNA and facilitate homologous recombination with genomic DNA that targets integration at a specific genomic locus. On the other hand, gene replacement has lower efficiency and generally an efficiently directed integration requires longer terminal flanking sequences (Lin-Cereghino & Cregg, 2000). Alternatively, it has been shown that episomal expression vectors (e.g. pGAPZ-E) may offer advantages over integration vectors as they can be easily transferred from strain to strain, and they do not require integration procedure and multi-copy screening (Hong *et al.*, 2007).

1.3.5. Placental alkaline phosphatase (PLAP) recombinant protein

PLAP is a human soluble protein coded by a 1.53 kb gene and having a molecular weight ranging from 65 kDa to 75 kDa. This protein contains no O-linked glycosylation sites and a single occupied N-linked site (Schlaeger *et al*., 2003, Zhang *et al*., 2000). Alkaline phosphatases are dimeric Zn^{2+} and Mg^{2+} binding metallozymes that are able, under alkaline conditions, to hydrolyse phosphodiester bonds. Alkaline phosphatases (AP) are found in nature in almost all living organisms, and they have different physiological functions within different organisms, as well as within a single organism. For instance, AP are found in *E. coli* as soluble, periplasmic proteins or they are found in mammalian cells bound to the cell surface (Heimo *et al*., 1997).

In this study a variant of the human placental alkaline phosphatase (PLAP, accession number NM_001632) which has had a lipid anchor signal deleted was used (Cullen and Malim, 1992). This variant has been referred to as hPLAP. A variant of hPLAP in which the original human leader peptide sequence is replaced with the cleavable signal sequence of the small peptide mating pheromone from Saccharomyces cerevisiae strain, MAT alpha, was also generated (Waters *et al*., 1988). This variant has been referred to as αPLAP.

The hPLAP and αPLAP variants may have different properties with respect to their interactions with the *P. pastoris* secretory pathway. Specifically, preliminary observations (data not shown) suggest these recombinant proteins can be detected in the soluble interior and membranes of *P. pastoris* cells, as well as secreted to the growth medium. An investigation of the intracellular trafficking and secretion of these proteins is ongoing by others, both in terms of findings and assay development, and falls outside the scope of this study. As such, all characterisation of αPLAP and hPLAP productivity in this thesis focuses solely on total volumetric productivity, or total volumetric productivity as a function of cell density (Specific yield).

As stated above, there are some heterogeneities in the molecular weight (MW) and structures of some AP. There are two main reasons for this phenomenon: remarkable phenotypic variation in mammalian cells (allelic variation causes differences on AP activity, more details can be found in Lucarelli *et al.* (1982)) and heterogeneous post-translational modifications. These sources of variation in AP are a potential drawback for commercial use, which coupled with low production titres, creates the need of novel culturing methods aimed to improve protein homogeneity and concentration. Therefore,

P. pastoris was identified as a possible host candidate for the production of these enzymes, as it offers the possibility of large scale production of recombinant protein to several grams per litre as well as a post-translational processing similar to higher eukaryotes (Heimo *et al.*, 1997).

1.4. Pichia pastoris downstream processing (DSP)

Usually, downstream process protocols require multiple steps and complex designs to obtain highly purified proteins. An ideal protocol will, however, achieve the same outcome with minimal steps and simple design (Li *et al.*, 2009). Because of the low secretion of homologous proteins by *Pichia pastoris*, protein purification is eased. In fact, in this system the amount of secreted recombinant protein can reach over 95% of the total protein in the culture medium (He *et al.*, 2009).

The high-cell-density (over 150 g/L dry cell weight and up to 50% wet weight cells) achieved by *Pichia pastoris* systems causes complications in downstream processing. In addition, conventional standard bioreactor medium has a high salt concentration which ranges from 40 mS/cm at the start of the cultivation to 15-20 mS/cm at the end. At the time this medium was designed (1970s), *Pichia pastoris* was mainly used for single cell protein production, which does not require advanced downstream process techniques. This medium also ease accumulation of lipid-like substances, aggregation of recombinant proteins, high osmotic pressure in the medium leading to cell lysis and death (Brady *et al.*, 2001; Cheroenrat *et al.*, 2006; Wegner, 1983).

Solid-liquid separation is usually performed by centrifugation and filtration. For scaled-up processes continuous centrifugal separators are available. However, the disk-stack separator is usually limited to 30% of maximum wet weight cells. For higher wet weight cells (up to 50%) decanter centrifuges are used, but separation efficiency is lower. Thus, additional washing steps are required in order to recover the most of the entrapped product (Axelsson, 2000).

1.4.1. High cell density solid-liquid separation

Cross-flow microfiltration (0.45 μm) in diafiltration mode allows for better separation of 50% wet weight cells culture broth. Increased downstream process efficiency can be increased by integrating the cultivation and downstream processes. Two candidate techniques are expanded bed adsorption (EBA) and partitioning in aqueous two-phase systems (ATPSs). EBA allows to directly and cost-effectively recover the target protein from the crude feedstock in a one-step procedure. On the other hand, ATPSs do not compromise the resolution capacity for suspensions up to 50% wet weight cells. In addition, scaling-up and continuous operations are feasible, but an additional ion exchange chromatography step is needed. Finally, it is suggested that higher wet weight cells perform better with ATPSs, and that EBA is preferred for intermediate cell densities (up to 12% wet weight cells) chemostat cultures because of a five times dilution need prior to EBA operations required to reduce salt concentration, density, and viscosity. Recent advances offer a wider range of possibilities. For instance, GE Healthcare (Uppsala, Sweden) developed a new inlet system with higher bead density that allows

working at higher feedstock concentration and higher flow velocity. In addition, new media with lower salt concentration ease the interaction with EBA operation, while improving cell viability and productivity. These advances allow nowadays to reach yields of 95% and purities up to 90% within a one-step product recovery (Jahic *et al.*, 2006).

1.4.2. Typical purification method

A typical method for the purification of secreted proteins involves four main steps. First, harvested cells are separated from the broth through a centrifugation step. Then, the cell-free broth is diluted 1:1 (v/v) with 50 mM sodium acetate (NaAc) pH 5. The diluted broth is then passed through an ion-exchange column equilibrated with NaAc (sodium acetate) buffer. The column is then washed with NaAc. The product is eluted with a linear gradient from 0 to 1M NaCl in NaAc buffer. At this stage of purification, the protein is typically 90% pure or greater. A sometimes-useful third step of purification is a hydrophobic interaction chromatography (HIC). Because the post-ion-exchange column material is already in high salt, little treatment is required to prepare it for application to a HIC column. Additional salt (NH_4SO_4) is added to the solution to achieve 40 to 50% saturation. The solution is then passed through a column containing an appropriate HIC resin. Phenyl and butyl HIC resins have been used successfully, but this varies from protein to protein. The product is eluted from the column by a decreasing linear gradient from 40% to 0% of NH_4SO_4. After elution from the HIC resin, the product purity generally is greater than 95% and after dialysis and lyophilisation it appears as a white

powder. Both ion-exchange and HIC chromatography steps have been proven effective and can be scaled up to accommodate larger volumes. Additional down-stream chromatography steps may be needed to improve purity or to separate monomer forms from multimers of the same molecule (Cregg *et al.*, 1993).

1.5. Pichia pastoris *in Industry*

1.5.1. Emerging technologies

Usually, *Pichia pastoris* expression systems are under the control of a single, inducible promoter. Duan *et al.* (2009) showed that two promoters, $\boldsymbol{P}_{AOX1}$ and $\boldsymbol{P}_{FLD1}$, can work together in a single vector. These promoters can both be induced simultaneously by methanol, but they can be induced at different times, too. This is possible because the *FLD1* promoter is also induced by a combination of methylamine and glucose or sorbitol. The combined use of these promoters has been described as being cost and time effective as it can control the co-expression of two foreign genes in a host cell (e.g. the host cell do not naturally express an enzyme necessary for the correct folding of the target protein), control the gene product concentration, and allowing for co-existence studies (e.g. how two proteins interact and influence themselves) (Duan *et al.*, 2009). However, to our knowledge, no studies so far showed the effectiveness of using a dual-promoter vector for the expression of a single recombinant protein.

It has been shown that Mut^- strains produce higher amounts of recombinant protein than Mut^+ when growth on non-repressing substrates such as alanine, mannitol, sorbitol, threhalose, and lactic acid. A comparison between methanol/glycerol and

methanol/sorbitol fed-batch mixed feed strategies showed that final production levels are comparable as methanol/sorbitol produced lower amounts of biomass, but higher specific product formation rates. Not being a repressing substrate, sorbitol offers great production advantages since tight control of substrate accumulation is no longer necessary, leading to work at non-limiting feed conditions. Furthermore, lower amounts of methanol are to be used as sorbitol supports almost the totality of cell growth. Table 1.6 shows a comparison among different mixed substrate strategies (Sreekrishna *et al.*, 1997; Thorpe *et al.*, 1999).

Table 1.6: Mixed substrates culture comparison

Protein	Phenotype	Co-substrate	Feeding strategy		μ 1/h (gDCW/L h)*	Production mg/L (μmol/L)*	Productivity mg/L h (μmol/L h)*	Specific Productivity mg/g h (μmol/g DCW h)*
Immunotoxin A-dmDT390-bisFv(G4S)	Mut^+	Glycerol	Methanol:Glycerol 4:1		----	37	0.55	----
mAb4813	Mut^+	Glycerol	Methanol constant at 5 g/L	Constant glycerol feeding rate at 4.90 g/L h	0.023	~0	----	----
				Constant glycerol feeding rate at 2.46 g/L h	0.016	~0	----	----
				Constant glycerol feeding rate at 1.23 g/L h	0.007	25	0.28	----
				No glycerol	0.012	45	0.52	----
Angiostatin	Mut^S	Glycerol	Methanol controlled at 5 g/L glycerol function of OD		0.012	108	1.66	0.019
		Sorbitol	Methanol controlled at 0.5 g/L sorbitol pre programmed at constant μ		0.018	141	2.76	0.030
		Lactic acid	Methanol controlled at 0.5 g/L lactic acid pre-programmed		0.011	191	2.96	0.044
Cystatin-C	Mut^S	Glycerol	Constant methanol feeding rate 1.8 g/L h	constant glycerol feeding rate at 2.1 g/L h	0.48*	45*	0.60*	0.008*
				constant glycerol feeding rate at 3.5 g/L h	0.81*	34*	0.96*	0.009*
				constant glycerol feeding rate at 6.4 g/L h	1.38*	15*	0.95*	0.008*
				No glycerol	0.08*	54*	0.60*	0.011*
Sea raven AFP	Mut^S	Glycerol	Continuous		0.01	60	0.6	0.015
					0.09	15	1.6	0.065
			Fed-batch at constant μ = 0.03 1/h		0.03	180	2.3	0.04
			Fed-batch at constant μ = 0.07 1/h		0.07	120	2.4	0.04
		Glycerol	Methanol constant at 1-2 g/L	Fed-batch	----	180	1.5	0.045
		Sorbitol		Fed-batch	----	200	1.7	0.060
rFSH	Mut^S	Only methanol	pre-programming feeding fed-batch		----	45	0.3	0.014
		Glycerol			----	27	0.2	0.011
		Sorbitol	Continuous		----	187	1.3	0.035
		Only methanol			0.012	170	2.0	0.033
		Sorbitol	pre-programming feeding fed-batch		0.005	282	1.4	0.018
					0.01	121	1.1	0.018
β-galactosidase	Mut^-	Glycerol	Constant methanol	Constant glycerol feeding rate at 1 g/L h	----	415 U/mL	8.5 U/g h	7865 U/g h
				Constant glycerol feeding rate at 4 g/L h	568 U/mL	10.7 U/mL h	37.17 U/g h	----
				Constant glycerol feeding rate at 7 g/L h	577 U/mL	12.3 U/mL h	3005 U/g h	----
				Step increase glycerol feeding rate	340 U/mL	6.7 U/mL h	4392 U/g h	----

(Cos *et al.*, 2006)

Scale-up and optimisation of recombinant proteins production is usually based on trial and error assays. In industry, 'Design of Experiments' (DoE) often helps to rationalise experiments by looking at factors affecting production yield. These factors are usually experimental set-up parameters such as medium composition, nutrient feed rates, and induction of expression methods. A DoE-derived model has been shown to facilitate the scale-up process. Parallel mini-bioreactor, having pH, temperature, and dissolved oxygen probes, is predictive for culture volume, density, and stepwise scale-up. Each of the factors taken into consideration (pH, temperature, and dissolved oxygen) is tested at three levels (low, middle, and high values) in order to generate predictive models through statistical software. The three on-line input factors are kept constant during 'fermentation' in micro-wells (M24 system), while measurable off-line outputs (OD, specific yield, and protein assay) are analysed. Each input factor is then plotted against the output ones, and a factorial analysis based on Eigen-values will determine the influence that factors have in relation to each others, generating a three-parameter equation (Holmes *et al*., 2009).

Recently, the first *Pichia pastoris* genome sequence has been published (De Shutter *et al.*, 2009). This tool will provide the scientific community with a web-based easy-to-access database to speed-up research. In fact, DNA microarrays, metabolic modelling, recombination events, and other applications will be facilitated. In this context, there are two main databases available. The former was set-up at http://www.pichiagenome.org (GBrowse based genome browser) and it is based on the *Pichia pastoris* DSMZ 70382 ('Wild' *Pichia pastoris*) genome. The latter is a genome portal with gene annotation and browsing functionality

(http://bioinformatics.psb.ugent.be/webtools/bogas) based on *Pichia pastoris* GS115 from Invitrogen Corporation. Gene annotation, function, regulation, and structure information is offered on both websites (Mattanovich *et al.*, 2009).

1.5.2. Intellectual Property (IP) limitations and freedom to operate

Pichia expression systems from Invitrogen Corporation have been developed by Salk Institute Biotechnology/ Industry Associates (SIBIS) and Phillips Petroleum. All patents (Table 1.7) and licences protecting these products are owned by Research Corporation Technologies (RCT), Inc., Tucson, Arizona, USA. With its products Invitrogen Corporation sells a non-exclusive license for academic research or for evaluation purposes only. Any further use is subject to a license request to RCT (http://www.invitrogen.com).

Table 1.7: Invitrogen's *Pichia pastoris* patents
Patents that protect Invitrogen's products and expected expiry dates. Note that these are US patents, which are owned, including corresponding foreign patents, by Research Corporation Technologies (http://www.freepatentsonline.com; http://www.invitrogen.com). Relevance: At present there are no patents limitating the use of *P. pastoris* in the frame of this project.

Patent number	Filing date	Expected expiry	Title	Inventors
US4683293	20/10/1986	20/10/2006	Purification of *Pichia* produced lipophilic proteins	Craig
US4808537	30/10/1984	30/10/2004	Methanol inducible genes obtained from *Pichia* and methods of use	Stroman, Brust, Ellis, Gingeras, Harpold, Tschopp
US4812405	18/02/1986	18/02/2006	Double auxotrophic mutants of *Pichia pastoris* and methods for preparation	Lair, Digan
US4818700	25/10/1985	25/10/2005	*Pichia pastoris* argininsuccinate lyase gene and uses thereof	Cregg, Sperl
US4837148	30/10/1984	30/10/2004	Autonomous replication sequences for yeast strains of the genus *Pichia*	Cregg
US4855231	25/09/1985	25/09/2005	Regulatory region for heterologous gene expression in yeast	Stroman, Brust, Ellis, Gingeras, Harpold, Tschopp
US4857467	23/07/1986	23/07/2006	Carbon and energy source markers for transformation of strains of the genes *Pichia*	Sreekrishna, Fuke
US4879231	30/10/1984	30/10/2004	Transformation of yeasts of the genus *Pichia*	Stroman, Cregg, Harpold, Sperl
US4882279	21/11/1989	21/11/2009	Site selective genomic modification of yeast of the genus *Pichia*	Cregg
US4885242	30/10/1984	30/10/2004	Genes from *Pichia* histidine pathway and uses thereof	Cregg
US4895800	26/11/1985	26/11/2005	Yeast production of hepatitis B surface antigen	Tschopp, Harpold, Cregg, Buckholz
US4929555	19/10/1987	19/10/2007	Pichia transformation	Cregg, Barringer
US5002876	22/09/1986	22/09/2006	Yeast production of human tumor necrosis factor	Sreekrishna, Fuke
US5004688	15/04/1988	15/04/2008	Purification of hepatitis proteins	Craig, Siegel
US5032516	24/06/1988	24/06/2008	*Pichia pastoris* alcohol oxidase II regulatory region	Cregg
US5122465	12/06/1989	12/06/2009	Strains of *Pichia pastoris* created by interlocus recombination	Cregg, Digan
US5135868	01/09/1989	01/09/2009	Cultures of yeast of the genus *Pichia* altered by site selective genomic modification	Cregg
US5166329	07/02/1992	07/02/2012	DNA encoding the alcohol oxidase 2 gene of yeast of the genus *Pichia*	Cregg

1.5.2.1. Patent law

Usually, standard patents last 20 years from the filing date in a chosen country (jurisdiction). Patent infringement can only be enforced if it violates exclusive rights in such jurisdiction. Therefore, patents must be filed in all countries where such a right want to be protected. Alternatively, an international agreement allows filing international patents in order to reduce costs and time. Pharmaceutical-related patents are sometimes granted an up to five years extension aimed to cover times required to claim regulatory approval. Once the patent term has expired, such knowledge becomes part of the public domain, and anyone is able to freely exploit the invention (Fitzgerald & Fitzgerald, 2004).

1.5.2.2. Other patents

Besides RCT's owned intellectual property, other patents may restrict the freedom to operate using *Pichia pastoris* expression systems. In Table 1.8 below is reported a list of granted patents in this subject area (http://appft.uspto.gov). However, none of those seem to restrict freedom to operate in the actual frame of this project as there are no commercial objectives at present.

Table 1.8: *Pichia pastoris* patents
Granted and pending patents related to the *Pichia pastoris* expression systems (http://appft.uspto.gov).

Patent number	Filing date	Expected expiry	Title	Inventors
20080153126	23/02/2006	23/02/2026	Mutant *AOX1* promoters	Hartner, Glieder
20070298500	04/06/2007	04/06/2027	Formaldehyde dehydrogenase genes from methylotrophic yeasts *	Cregg
7572616	01/11/2006	01/11/2026	Alternative oxidase and uses thereof	Rustin, Jacobs, Dassa, Hakkaart, Fernandez-Ayala, Moreno
7250502	12/06/2003	12/06/2023	Formaldehyde dehydrogenase genes from methylotrophic yeasts *	Cregg
6730499	02/06/1999	02/06/2019	Promoter for the *Pichia pastoris* formaldehyde dehydrogenase gene *FLD1* *	Cregg
6410264	31/07/1998	31/07/2018	*Pichia pastoris* gene sequences (PpSEC10) and methods for their use	Crawford, Bishop
5641661	25/03/1994	25/03/2014	*Pichia pastoris* alcohol oxidase *ZZA1* and *ZZA2* regulatory regions for heterologous gene expression	Kumagai, Sverlow
5032516 **	24/06/1988	24/06/2008	*Pichia pastoris* alcohol oxidase II regulatory region	Cregg
5500483	25/03/1993	25/03/2013	*Pichia pastoris* alcohol oxidase *ZZA1* regulatory region for heterologous gene expression	Kumagai, Sverlow
5665600**	18/09/1991	18/09/2011	*Pichia pastoris* linear plasmids and DNA fragments thereof *	Hagenson, Barr, Stroman, Gaertner, Harpold, Klein

* Assigned to RCT; ** Expired (at Sep. 2012).

1.6. Aims and objectives

It was shown in previous sections that *Pichia pastoris* expression systems are widely used for the production of heterologous proteins. However, some work still needs to be done to optimise such systems. The effects of methanol feed strategy was tested across four experimental strains, Mut^+ and Mut^S strains each expressing a PLAP variant with its original human signal peptide (hPLAP) and Mut^+ and Mut^S strains each

expressing a PLAP variant with a yeast signal peptide (αPLAP). The different signal peptides are predicted to effect differing nature and degrees of interaction with the *P. pastoris* secretory pathway. To capture a global picture of how carbon source feeding influences productivity, the focus on how these experimental strains differ in their overall productivity of each reporter protein was investigated. Other than studying those cells and the protein production outcome at a genetic level, in this project those systems were optimised also a culture level. A 3-steps optimisation was performed. First, a biomass growth optimisation was done with the objective of being able to produce biomass faster and to higher levels. Then, tests were performed to optimise to test cell reaction in productivity levels with different methanol flow rates. Finally, the two outcome methods were combined with the intent to boost PLAP titres, and therefore answer the question: Is *Pichia pastoris* able to over-produce recombinant proteins only by changing the methanol feed strategy? Another question that was tried to give an answer in this project was: Can simple genetic modifications (changing the leader sequence) increase volumetric and specific yield of *Pichia pastoris* expression systems? The objectives, each corresponding to one chapter, drawn within this project and experimentally investigated are listed below.

Objective 1: Investigate sensitivity of a DHAA-based assay for at-line methanol monitoring (Chapter 3)

Methanol concentration is a key parameter for *Pichia pastoris* cultivation. However, methanol probes are expensive, and common assay are sometime impractical or too laborious (e.g. long reaction times). Therefore, a novel assay was created.

Objective 2: Compare suitability of mechanistically distinct alkaline phosphatase activity assays for off-line PLAP monitoring (Chapter 3)

Two mechanistically different PLAP assays were tested and optimised in order to monitor PLAP production during all the different culture methods used in this work.

Objective 3: Construction and verification of *P. pastoris* PLAP variant overexpressor strains (Chapter 4)

It in project it was sought to create four new *P. pastoris* strains using the pAO36 and pPSS plasmids systems. Both Mut^{+} and Mut^{S} phenotype need to be created, where the Mut^{+} clones are created via homologous recombination, and the Mut^{S} strains are created via omega insertion. All new strains phenotypes need to be confirmed by selective growth and PCR.

Objective 4: Determine influence of methanol feed rate on biomass accumulation (Chapter 5)

Different methanol feed strategies were used and compared in terms of optical density (OD) and fermentation timescales.

Objective 5: Determine influence of methanol feed rate on specific yield of recombinant PLAP (Chapter 6)

It was investigated how the influence of methanol feed rate on biomass accumulation also affected PLAP productivity performance.

2. Material and methods

2.1. DNA preparation and analysis

2.1.1. DNA concentration and visualisation

2.1.1.1. DNA quantification by spectrophotometry

DNA quantification was performed in NanoDrop 1000 (Thermo Scientific) and analysed by the attached software. Blank was done by adding 1 µL ddH_2O to the reading chamber and 1 µL sample was then quantified at 280nm.

2.1.1.2. DNA analysis by Agarose Gel Electrophoresis

DNA samples (typical volume 20 µl) were ran in 1.5% Agarose (Sigma Aldrich Company, LTD, UK) gel made with 1xTBE (Tris-Borate-EDTA electrophoresis - Thistle Scientific LTD, UK) buffer. 2 µl Ethidium Bromide (Sigma Aldrich Company, LTD, UK) were added to the molten gel before pouring to visualise bands under UV light.

Gel preparation

1.5 g Agarose powder was dissolved in 100 mL 1xTBE buffer. The solution was microwaved until powder completely dissolved and liquid appeared clear. 2 µL Ethidium Bromide were added before pouring the solution in the running chamber. Gel was stored in a fume hood to set for 1-2 hours.

Samples preparation

8 µL sample were mixed with 2 µL loading dye (DNA Loading Buffer Blue 5x - Bioline Reagents LTD) and loaded into the gel. 1kb DNA ladder (Figure 2.1 - NEB – New England Biolabs, USA) was used to estimate DNA fragment sizes.

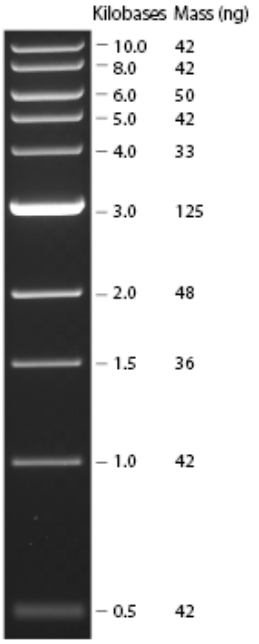


Figure 2.1: 1 kb DNA ladder profile
1 kb DNA Ladder visualized by Ethidium bromide staining on a 0.8% TAE Agarose gel. Mass values are for 0.5 µg/lane (New England Biolabs, USA).

Migration and UV visualisation

Gels were run for 2 hours at 100 Volts in TBE buffer using Biorad PowerPac Basic and DNA bands were visualised by Trans-UV light using Biorad Gel Doc 2000.

2.1.2. DNA – Analytical Scale

2.1.2.1. Purification of 0.1-1 µg of DNA

QIAquick® Gel Extraction Kit (50), Qiagen and Nucleic acid extraction kit (100), Anachem

DNA extraction was performed as outlined in the kits protocols. DNA was then quantified by NanoDrop 1000 and band sizes visualised by Electrophoresis gel.

2.1.2.2. Restriction digests of 100-500 ng of DNA

Enzymes

All the enzymes (and joint buffers) were obtained commercially from New England Biolabs (Ipswich, MA, USA) and they were *Eco*RI, *Pst*I, *Bgl*II, *Sal*I, CIP, and Quick Ligase (T4).

Typical single-enzyme digestion protocols

Mini: Sample composition was 1 µL enzyme, 3 µL DNA, and 1 µL 5x Buffer. Reaction tubes were incubated at 37°C for 2 hours at 250 rpm. Where necessary enzymes were inactivated by incubation at 65ºC for 20 min. Digestion mixture was visualised by electrophoresis gel to confirm complete digestion of the samples.

Medium: Sample composition was 2 µL enzyme, 35 µL DNA, 10 µL 5x Buffer, and 3 µL ddH_2O. Reaction tubes were incubated at 37°C for 2 hours at 250 rpm. Where necessary enzymes were inactivated by incubation at 65ºC for 20 min. Digestion mixture was visualised by electrophoresis gel to confirm complete digestion of the samples.

Typical double-enzyme digestion protocol

Medium: Sample composition was 2 µL enzyme 1, 2 µL enzyme 2, 35 µL DNA, 10 µL 5x Buffer, and 1 µL ddH_2O. Reaction tubes were incubated at 37°C for 2 hours at 250 rpm. Where necessary enzymes were inactivated by incubation at 65ºC for 20 min. Digestion mixture was visualised by electrophoresis gel to confirm complete digestion of the samples.

2.1.3. DNA – Preparative Scale

2.1.3.1. Purification of 1-10 µg of DNA

HiSpeed® Plasmid Maxi Kit (10), Qiagen

DNA extraction was performed as outlined in the kit protocol. DNA was then quantified by NanoDrop and visualised by Electrophoresis gel.

2.1.3.2. Restriction digests of 0.1-20 µg of DNA

Typical single-enzyme digestion protocols

Mini: 1 µL enzyme, 5 µL DNA, 1 µL 10x Buffer, 3 µL ddH_2O

Reaction tubes are incubated at 37°C for 2 hours at 250 rpm. Where necessary enzymes are inactivated by incubation at 65ºC for 20 min. Digestion mixture is visualised by electrophoresis gel to confirm complete digestion of the sample.

Maxi: 10 µL enzyme, 100 µL DNA, 15 µL 10x Buffer, 25 µL ddH_2O

Reaction tubes are incubated at 37°C for 2 hours at 250 rpm. Where necessary enzymes are inactivated by incubation at 65ºC for 20 min. Digestion mixture is visualised by electrophoresis gel to confirm complete digestion of the sample.

Typical double-enzyme digestion protocol

Maxi: 20 µL enzyme 1, 20 µL enzyme 2, 200 µL DNA, 30 µL 10x Buffer, 30 µL ddH_2O

Reaction tubes are incubated at 37°C for 2.5 hours at 250 rpm. Where necessary enzymes are inactivated by incubation at 65ºC for 20 min. Digestion mixture is visualised by electrophoresis gel to confirm complete digestion of the sample.

2.1.4. Ligations

Typical ligation protocol - 2 fragments ligation

Ligations were performed in double using different molar ratios (1:1 and 1:3) and controls (single fragment recircularisation in absence and presence of ligase).

1:1

1 µL Quick ligase (T4) was mixed with 2.5 µL fragment 1, 2 µL fragment 2 (plasmid backbone), 4.5 µL ddH_2O (ratios vary according to DNA molar concentration) and 10 µL 2x Buffer. Reaction tubes were incubated at room temperature for 20 minutes and then frozen overnight. Ligation mixture was then, were necessary, purified, and used for cell transformation.

1:3

1 µL Quick ligase (T4) was mixed with 2 µL fragment 1 (plasmid backbone), 7 µL fragment 2 (ratios vary according to DNA molar concentration) and 10 µL 2x Buffer. Reaction tubes were incubated at room temperature for 20 minutes and then frozen overnight. Ligation mixture was then, were necessary, purified, and used for cell transformation.

DNA ligation reactions

In order to test ligation efficiency two controls have been used: Control 1 (C1) – single fragment in presence of ligase. The aim of this control was to confirm the efficiency of the digestion with phosphatase step, as where phosphate is removed, re-circularisation of the plasmid should not happen. Control 2 (C2) – single fragment in absence of ligase. In this case the importance of this control is to show that spontaneous plasmid recircularisation did not happen. The controls were also used to estimate the number of transformed cells needed for the screen of positive clones as the ratio between controls and samples gave the amount of possible false positive transformants within a sample.

Typical ligation protocol - 3 fragments ligation

All fragments were diluted with ddH_2O in order to have the same DNA concentration in all of them. 1 µL Quick Ligase (T4) and 13 µL 2x buffer were mixed with 3 DNA fragments at different ratios: 1:1:1, 1:2:3, and 1:2.5:2.5. Reaction tubes were incubated at room temperature for 20 minutes and then frozen overnight. Ligation mixture was then, were necessary, purified, and used for cell transformation.

1:1:1

4 µL of each fragment were used for this reaction.

1:2:3

2 µL backbone (fragment 1) were mixed with 6 µL fragment 2 (smallest fragment) and 4 µL of fragment 3.

1:2.5:2.5

2 µL backbone (fragment 1) were mixed with 5 µL fragment 2 and 5 µL of fragment 3.

2.1.5. Plasmid DNA (pDNA) identification

2.1.5.1. Polymerase Chain Reaction (PCR)

Typical PCR reaction composition

5 µL 10x Standard Taq Buffer (containing $MgCl_2$) were mixed with 1 µL 50x dNTPs (dATP, dCTP, dGTP, and dTTP), 1 µL forward primer, 1 µL reverse primer, 1 µL DNA polymerase (*Taq* PCR kit, Cat. No. E5000S, New England Biolabs, UK), 1 µL DNA, and 40 µL ddH_2O.

Typical PCR reaction parameters

Veriti 96 wells Thermo Cycler (Applied Biosystemsm, Life Technologies, Carlsbad, USA) was used for DNA amplification with PCR method using the following parameters:

- Initial denaturation step: 1 min at 95°C
- Denaturation step: 30 seconds at 95°C
- Primer annealing step: 45 seconds at 60°C
- Extending step: 50 seconds at 72°C
- Cycles: 35
- Final extension step: 5 minutes at 72°C
- Hold: 4°C

After PCR samples were visualised by electrophoresis to determine PCR outcome.

2.1.5.2. Sequencing

DNA sequencing has been performed for us by the Wolfson Institute, University College of London, UK.

2.1.5.3. DNA precipitation and purification

Linearised DNA and ligation mixture were precipitated and purified by adding 0.1 volumes of 3M sodium acetate (Sigma Aldrich Company LTD, UK) and 2.5 volumes of 100% ethanol (Sigma Aldrich Company LTD, UK), vortexed, and DNA was precipitated overnight at -80˚C. Following, samples were spun 20 minutes at 12000 rpm at 4˚C, supernatant was discarded and speed-vacuumed at room temperature for 10 minutes. Pellets were resuspended in 20 μl TE buffer (Qiagen).

2.1.6. Transformation and cryopreservation of *Pichia pastoris*

2.1.6.1. *Pichia pastoris* transformation by electroporation

Electro-competent cells preparation

A colony of *Pichia pastoris* GS115 WT was grown in 5 ml YPD broth at 30˚C overnight. 500 ml of fresh YPD broth were then inoculated with 0.5 ml of the overnight culture and grown overnight at 30˚C until OD_{600} = 1.3 – 1.5. Cells were centrifuged at 1500 x g for 5 minutes at 4˚C. The pellet was resuspended in 500 ml of ice-cold, sterile water. Centrifugation was repeated and the pellet was resuspended in 250 ml of ice-cold, sterile water. The centrifugation procedure was repeated once more and cells were resuspended in 20 ml ice-cold 1M sterile sorbitol (Sigma Aldrich Company LTD, UK). Centrifugation was repeated one last time and the pellet was resuspended in 1 ml of ice-cold 1M sterile sorbitol. Cells were now electrocompetent.

Transformation

Pichia pastoris clones were generated through homologous recombination of a vector into the host's genome. pAO815 from Invitrogen Corporation (figure 1.9) is a vector specifically designed for the creation of His^+ Mut^+ and His^+ Mut^S phenotypes in *Pichia pastoris* GS115. The PLAP gene was put under the control of the strong *AOX1* by inserting it at the *Eco*RI site of the pAO815 plasmid. After transformation of the generated plasmid into *Pichia pastoris*, two strains were obtained, respectively, by linearization with *Sal*I or *Stu*I (insertion at *HIS4*), or by linearization with *Bgl*II (gene replacement at *AOX1*) (http://www.invitrogen.com).

Note: Ligation mixture containing PEG must be purified prior to electroporation as described above.

5-20 μL DNA were added to 80-95 μl of electrocompetent cells. Transformation mixture was transferred to an ice-cold 0.2 cm Electroporation cuvette and incubated on ice for 5 minutes. Cells were pulsed according to preset fungi parameters and 1 ml of ice-cold 1M sorbitol was added immediately. Cells were transferred to a sterile Eppendorf tube and, after 1 hour incubation at 30˚C, the content was spread onto MD-Agar plates and incubated at 30˚C for two days.

2.1.6.2. Quantification of *P. pastoris* methanol utilisation phenotype

Phenotype confirmation

After incubation Mut phenotype was checked by plating colonies on YPD, MD and MM plates divided in a grid of 16 squares (figure 2.2). All *Pichia pastoris* cells must grow on YPD plates, HIS4 transformants (i.e. plasmid integrated) should be able to grow on

minimal media without histidine supplementation (i.e. MD and MM). MM plates were used to discriminate between Mut^+ and Mut^S transformants (i.e. Mut^S strains grow at slower rate than Mut^+ strains).

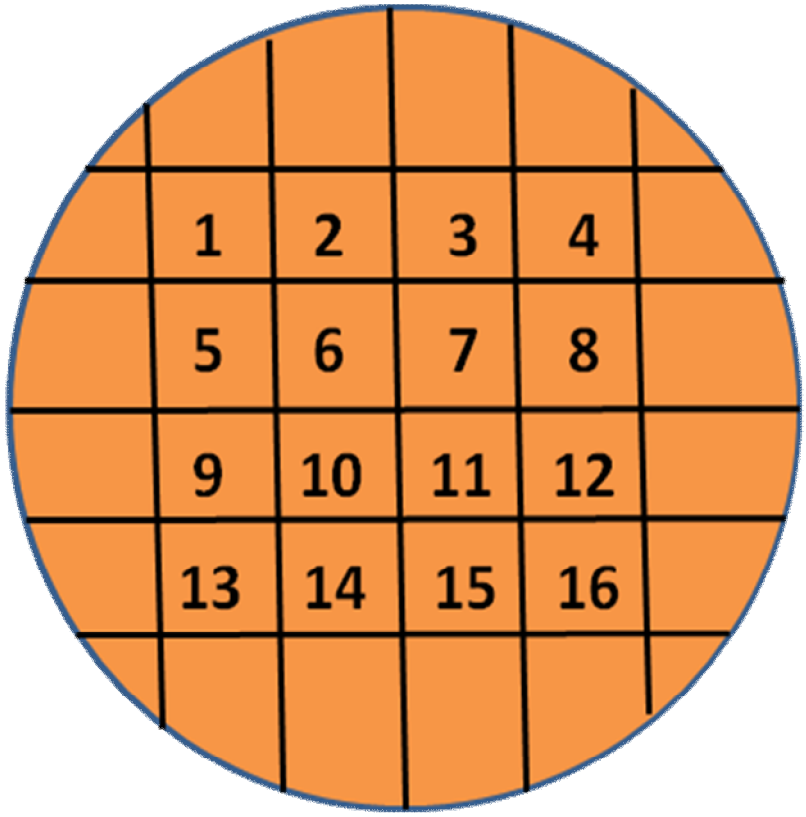


Figure 2.2: 16 squares grid for Petri dishes
16 squares grid template used for YPD, MD, and MM plates used for *P. pastoris* phenotype confirmation after transformation.

Genotype confirmation

PCR analysis was run to determine gene presence (i.e. integration). Genomic DNA extraction was performed either by heat (10 minutes at 95°C) or by sonication (6 seconds single pulse).

2.1.6.3. Generating *P. pastoris* Master and Working Cell Banks

After transformation 1 colony of the new strain was transferred to 5 mL of the desired media. Cells were grown overnight at 30°C at 300 rpm. Cells were then transferred to 49 mL fresh media and grown overnight using the same conditions. For small cell banks, 15 mL 80% sterile glycerol was added to the cells, which were then aliquoted (1 mL) into 1.2 mL cryotubes (PAA Laboratories ltd, UK). Cells were frozen overnight at -20°C and then transferred to -80°C for long term storage. 30 tubes were used as master cell bank (MCB), whereas the remaining tubes were used as the working cell bank (WCB). For larger cell banks the protocol outlined in figure 2.3 was followed.

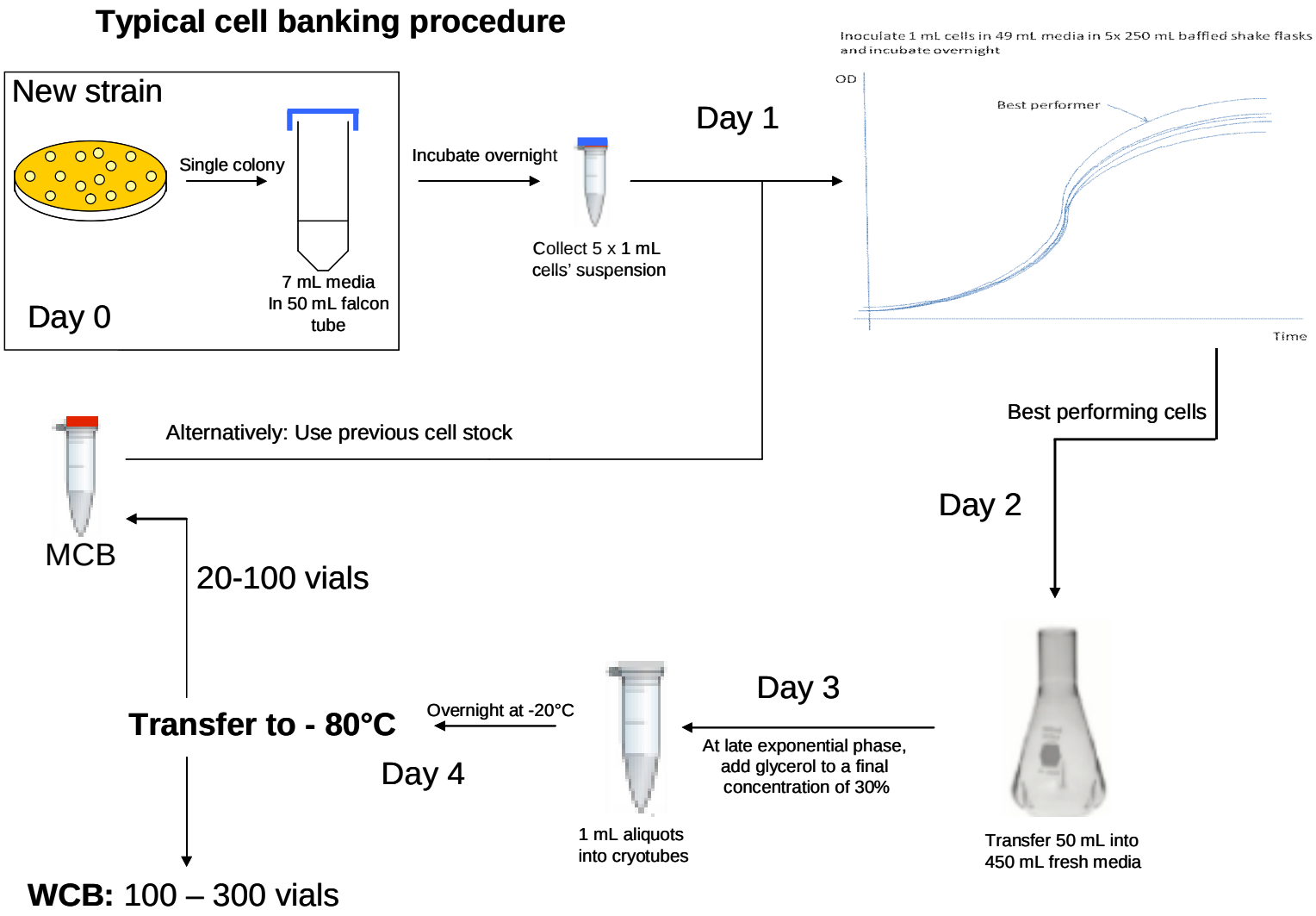


Figure 2.3: Typical cell banking procedure
After transformation, selected cells were grown overnight and aliquoted in 5x 1 mL tubes. Following, cells were grown in shake flasks. OD readings were taken at regular intervals and the best performing cells were used to generate the new strain's cell bank.

2.1.7. Transformation and cryopreservation of *E. coli*

2.1.7.1. *E. coli* transformation by heat shock

E. coli XL-10 Gold® Ultracompetent Cells (Stratagene, Cheshire, UK) were transformed accordingly to manufacturer protocol. 4 μL β-mercaptoethanol (β-ME) (Stratagene, Cheshire, UK) were added to 100 μL competent cells and incubated on ice for 10 minutes by swirling gently at regular intervals. 2 μL DNA were added and incubated once more on ice for 30 minutes. Samples were heat-pulsed for 30 seconds at 42°C and transferred again on ice for 2 minutes. 0.9 μL of preheated 42ºC LB broth were added to the mixture and incubated at 37°C for 1 hour with gentle shaking (250 rpm). 100 μL of heat-shocked cells were plated onto Petri dishes containing LB media supplemented with the desired antibiotic (e.g. Ampicillin).

Cells were then incubated overnight at 37ºC until colonies appeared. Single colonies were then grown in the same media (liquid form) before proceeding to cell banking and mini-preps to determine correct plasmid uptake.

2.1.7.2. Generating *E. coli* Master and Working Cell Banks

After transformation 1 colony of the new strain was transferred to 5 mL of the desired media. Cells were grown overnight at 37°C at 300 rpm. Cells were then transferred to 49 mL fresh media and grown overnight using the same conditions. For small cell banks, 15 mL 80% sterile glycerol was added to the cells, which were then aliquoted (1 mL) into 1.2 mL cryotubes (PAA Laboratories ltd, UK). Cells were frozen overnight at -20°C and

then transferred to -80°C for long term storage. 30 tubes were used as master cell bank (MCB), whereas the remaining tubes were used as the working cell bank (WCB). For larger cell banks the protocol outlined in figure 2.3 was followed.

2.2. Pichia pastoris Cultivation

2.2.1. *P. pastoris* Petri dishes media

Media Components

10xYNB (Yeasts Nitrogen Base) was prepared by adding 33.5g YNB (YNB with ammonium sulphate – Anachem LTD, UK) to 250 mL ddH_2O. This component was filter sterilised (TPP 250mL bottle top filter, PES, 0.22um, complete unit - Helena biosciences, UK) before being added to MD or MM media.

500xB was prepared by adding 20 mg D-Biotin (Fisher Scientific, UK) to 100 mL ddH_2O. This component was filter sterilised (0.22 μm, Millipore) before being added to MD or MM media.

10xM was prepared by adding 10 mL pure methanol (Fisher Scientific, UK) to 190 mL ddH_2O. This component was filter sterilised (TPP 250mL bottle top filter, PES, 0.22um, complete unit - Helena biosciences, UK) before being added to MM medium.

10xD was prepared by adding 40g D-glucose (Fisher Scientific, UK) to 200 mL ddH_2O. This component was autoclaved before being added to YPD and MD media.

YPD medium (YPD)

YPD medium was prepared by adding 10g Yeast Extract, 20g Peptone (Fisher Scientific, UK), and 20 g Agar to 900 mL RO water. After sterilisation by autoclave, 100mL 10xD were added to the medium, which was then poured into Petri dished and, once settled, stored at 4°C.

MD medium (MD)

MD medium was prepared by adding 15 g Agar to 800 mL RO water. After sterilisation by autoclave, 100 mL 10xYNB, 2 mL 500xB, and 100mL 10xD were added to the medium, which was then poured into Petri dished and, once settled, stored at 4°C.

MM medium (MM)

MM medium was prepared by adding 15 g Agar to 800 mL RO water. After sterilisation by autoclave, 100 mL 10xYNB, 2 mL 500xB, and 100mL 10xM were added to the medium, which was then poured into Petri dished and, once settled, stored at 4°C.

2.2.2. *P. pastoris* shake flasks media

Media Components

10xYNB, 500xB, 10xM, and 10xD were prepared as described in chapter 2.2.1.

10xGY was prepared by adding 20 mL 99% glycerol (Fisher Scientific, UK) to 180 mL ddH_2O. This component was autoclaved before being added to BMGY and BMMY media.

1M Potassium Phosphate buffer was prepared by mixing 132 mL 1M K_2HPO_4 with 868 mL 1M KH_2PO_4. pH was adjusted to 6.0 ± 0.1 with 1M KOH (VWR International LTD, UK). This solution was autoclaved to prepare BMMY and BMGY media.

1M K_2HPO_4 was prepared by adding 174.2 g K_2HPO_4 (MP Biomedicals, UK) to 1 litre RO water.

1M KH_2PO_4 was prepared by adding 136.1 g KH_2PO_4 (MP Biomedicals, UK) to 1 litre RO water.

YPD

YPD medium was prepared by adding 10g Yeast Extract and 20g Peptone to 900 mL RO water. After sterilisation by autoclave, 100mL 10xD were added to the medium, which was then stored at 4°C.

BMGY medium (BMGY)

BMGY medium was prepared by adding 10 g Yeast Extract and 20 g Peptone to 700 mL RO Water. After sterilisation by autoclave, 100 mL 1M Potassium Phosphate buffer, 100 mL 10xYPD, 2 mL 500xB, and 100mL 10xGY were added to the medium, which was then stored at 4°C.

BMMY medium (BMMY)

BMMY medium was prepared by adding 10 g Yeast Extract and 20 g Peptone to 700 mL RO Water. After sterilisation by autoclave, 100 mL 1M Potassium Phosphate buffer, 100 mL 10xYPD, 2 mL 500xB, and 100mL 10xM were added to the medium, which was then stored at 4°C.

2.2.3. *P. pastoris* fermentation media

BSM medium (BSM)

BSM medium for *P. pastoris* fermentation at 1 and 20 litres scales was prepared as outlined in table 2.1.

Table 2.1: BSM preparation for 1 and 20 litres scales

BSM preparation - Sterilised-in-place (SIP)			1L	20L
Volume needed		[L]	**0.45**	**7.20**
Phosphoric acid 85%	26.7 ml/L	[ml]	12.0	192.2
Calcium sulfate	0.93 g/L	[g]	0.42	6.70
Potassium sulfate	18.2 g/L	[g]	8.19	131.04
Magnesium sulfate $7H_2O$	14.9 g/L	[g]	6.71	107.28
Potassium hydroxyde	4.13 g/L	[g]	1.86	29.74
Glycerol (δ = 1261 g/L)	40 g/L	[g]	18	288
		[L]	0.014	0.228
Water	to 1 L	[L]	0.42	6.78

(Phosphoric Acid 85%, Calcium sulphate, potassium hydroxide - Sigma Aldrich Company LTD; Potassium sulphate - VWR International LTD, UK; Magnesium sulphate $7H_2O$ - Wilford Industrial Estate, UK; Glycerol – Fisher Scientific LTD). **Note:** Values are expressed for initial fermentation volume (volume needed) and not for the actual vessel volume (1 and 20 L).

Trace salts (PTM1)

PTM1 for *P. pastoris* fermentation at 1 and 20 litres scales was prepared as outlined in table 2.2.

Table 2.2: PTM1 preparation for 1 and 20 litres scales

PTM1 preparation - Filter sterilised			1L	20L
Volume needed (+10%)		[L]	**0.009**	**0.146**
Cupric sulfate $5H_2O$	6 g/L	[g]	0.055	0.874
Sodium iodide	0.08 g/L	[mg]	0.73	11.66
Manganese sulfate H_2O	3 g/L	[mg]	27.32	437.07
Sodium molybdate $2H_2O$	0.2 g/L	[mg]	1.82	29.14
Boric acid	0.02 g/L	[mg]	0.18	2.91
Cobalt Chloride	0.5 g/L	[mg]	4.55	72.85
Zinc Chloride	20 g/L	[g]	0.18	2.91
Ferrous sulfate $7H_2O$	65 g/L	[g]	0.59	9.47
Biotin	0.2 g/L	[mg]	1.82	29.14
Sulfuric acid	5 ml/L	[ml]	0.05	0.73
Water	to 1L	[ml]	9.1	145.0

(Sodium molybdate $2H_2O$, Cobalt chloride, Zinc chloride - Sigma Aldrich Company LTD; Boric acid - VWR International LTD, UK; Cupric sulphate $5H_2O$, Ferrous sulphate $7H_2O$, Magnesium sulphate H_20 - Wilford Industrial Estate, UK; Sodium iodide, Biotin, Sulfuric acid – Fisher Scientific LTD). **Note:** Values are expressed for initial fermentation volume (volume needed) and not for the actual vessel volume (1 and 20 L).

2.2.4. Yeasts strains

All yeasts that have been used in this project are listed in table 2.3.

Table 2.3: Yeasts used in this study

Microorganism	Strain	Origin
P. pastoris	GS115 wild-type (WT)	Invitrogen Corporation, Tucson, USA
P. pastoris	GS115 pPICZ lacZ	
P. pastoris	GS115 His^+ Mut^+ *β-gal*	
P. pastoris	GS115 His^+ Mut^- SEC HSA	

2.2.5. Vectors

All vectors that have been used during in this project are listed in table 2.4.

Table 2.4: Vectors used in this study

Vector	Source
pAO815 (figure 1.9)	Invitrogen Corporation, Tucson, USA
pSEAP2-control (figure 2.4)	Clontech Laboratories, Inc., Saint-Germain-en-Laye, FR
pRIS	Previously available at UCL, Department of Biochemical engineering, University College of London, London, UK

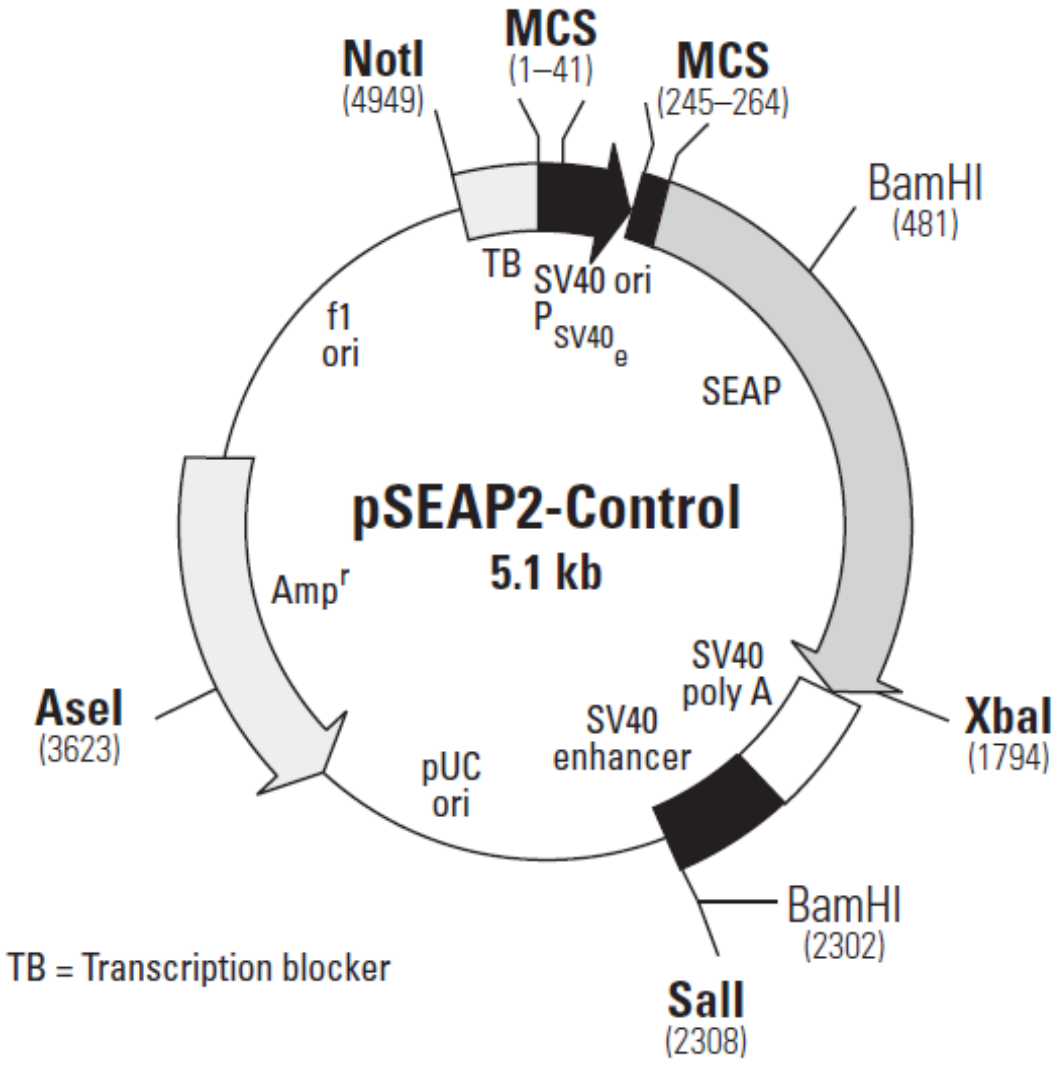


Figure 2.4: pSEAP2-control vector map
This vector has been used for the successful transient expression of placental alkaline phosphatase (PLAP) in HeLa cells.

2.2.6. *Pichia pastoris* 1L and 20L Fermentation inoculum

1 mL glycerol cell stock from WCB was added to 49 mL BMGY. Cells were incubated overnight at 30˚C and 250 rpm. For 20L fermentation only cells were then transferred to 450 mL BMGY and grown overnight under the same conditions until OD_{600} = 44 - 48.

2.2.7. *Pichia pastoris* Fermentation (1L and 20L)

Pichia pastoris fermentations were performed in an Applikon 20 L vessel and into Multifors 1L fermenters according to the conventional feed method, that could be found on-line at http://tools.invitrogen.com/content/sfs/manuals/pichiaferm_prot.pdf. In the downloadable file there is the full description of the standard *Pichia pastoris* fermentation method, including media recipes, feed-rates, and fermentation parameters.

500 mL of fermentation inoculum were added to 7.5 L BSM containing 12 mL/L PTM1 trace salts after proper in place 20L vessel sterilisation (SIP). 50 mL of fermentation inoculum were added to 0.5 L BSM containing 12 mL/L PTM1 trace salts after proper 1L vessel sterilisation.

Initial parameters were set as temperature 30°C, pH 6.5 (adjusted with ammonia 25% (v/v) (VWR International LTD, UK) and phosphoric acid 30% (v/v) (VWR International LTD, UK), DOT 30% (automatic cascade control), air supply 1 vvm. During fermentation 40% oxygen (BOC LTD, UK) and pure oxygen (BOC LTD, UK) were used as required. Glycerol fed-batch and methanol fed-batch were also performed as outlined in the conventional protocol.

However, the methanol feed was increased according to the DOT probe reading of the fermenter. DOT spikes and/or DOT increases were followed by a flow rate increase. Air flow rate during methanol fed-batch phase was kept constant with cascade control on pure oxygen. Fermentation broth was harvested after about 40-50 hours in methanol at a final volume of about 15 litres (20L) or 800 mL (1L).

20 mL samples were taken at regular intervals throughout the culture and especially during transition phases between Glycerol batch, Glycerol fed-batch, and methanol fed-batch. pH and OD readings were taken immediately, as were the measures of cell weights and microscope counting. Protein assays, however, were done after the culture was ended.

2.2.8. *Pichia pastoris* shake flasks and microwells growth

1 mL glycerol cell stock was added to 19 mL BMGY and grown overnight at 30˚C and 250 rpm. Cells were then transferred into 180 mL BMGY and grown under the same conditions until OD 600 nm ≈ 30. Following, the cell's suspension was centrifuged at max speed for 5 minutes and the pellet resuspended in sterile water in order to remove residual glycerol. The centrifugation step was repeated and cells were resuspended in BMMY medium to an OD_{600} ≈ 1-4. Cells were then aliquoted into shake flasks (200 mL/flask) or microwells (3mL/well in a 24 square-wells plate) and grown at 30˚C and 250 rpm for 72 hours. 99% methanol was added every 24 hours to the desired concentration (0.5 - 1%).

2.2.9. Optical density

Cell growth in a culture was assessed by optical density at 600nm (OD_{600}). 1 mL of cells was placed in a spectrophotometer cuvette and the absorbance was recorded. The reading was taken between 0.3 and 0.6 OD units. If higher, serial dilutions were made to fall within the reading range. The final OD value was the multiplication of the reading by the dilution. Samples were blanked versus the media used.

2.2.10. Cell number and viability

Cells were counted via microscope using a haemocytometer chamber (C-Chip Neubauer – PAA Laboratories LTD, UK). Viable cells were quantified via Trypan Blue 0.4% stain (VWR International LTD, UK). Viability was calculated by dividing the number of viable cells by the total number of cells.

2.2.11. Dry and wet cell weights

Wet cell weight (WCW) was obtained by centrifuging at max speed for 5 minutes 1 mL of cell suspension and discarding the supernatant. The difference between the tare of the preweighted Eppendorf tube and the pellet weight was the wet cell weight. The dry cell weight was obtained by placing the pellet obtained above at 90˚C overnight. The difference between the total weight and the tube tare was the dry cell weight (DCW).

2.2.12. pH

pH calibration was done by using pH 4.0 and 7.0 standards (pH calibration solutions, Mettler Toledo, UK). Readings were taken by inserting the pH probe (Gel Electrolyte pH

electrode, 0-14, InPro3030, Mettler Toledo, UK) into the media/solution to be assessed for off-line measurements or connected to the fermentation vessel for on-line measurements.

2.3. E. coli cultivation

2.3.1. *E. coli* media

LB (solid and liquid)

Liquid LB medium was prepared by adding 10 g/L Sodium Chloride (Fisher Scientific, UK), 10 g/L Tryptone (Fisher Scientific, UK), and 5 g/L Yeast Extract (Fisher Scientific, UK) to 1 litre of reverse osmosis (RO) water. The medium was then autoclaved and stored at 4°C. Solid LB media was prepared by adding 15g/L Agar (Fisher Scientific, UK) to the liquid LB medium before autoclaving. Liquid media was left to cool for about 20 minutes before pouring it into Petri dishes. Antibiotic were used as needed with both media by adding either 50-100 µg/mL Ampicillin (Sigma Aldrich Company LTD, UK) or 25-50 µg/mL Kanamycin (Sigma Aldrich Company LTD, UK) to the medium after sterilisation. Both antibiotics were dissolved in RO water as 1000x stock solution, filter sterilised (0.22 µm, Millipore), and stored at -20°C.

2.3.2. *E. coli* shake flasks growth

1 mL glycerol cell stock (*E. coli* XL-10 Gold® Ultracompetent Cells, Stratagene, Cheshire, UK) was added to 49 mL LB broth containing the desired antibiotic and cells were grown overnight at 37˚C and 350 rpm.

2.3.3. Optical density

Cell growth in a culture was assessed by optical density at 600nm (OD_{600}). 1 mL of cells was placed in a spectrophotometer cuvette and the absorbance was recorded. The reading was taken between 0.3 and 0.6 OD units. If higher, serial dilutions were made to fall within the reading range. The final OD value was the multiplication of the reading by the dilution. Samples were blanked versus the media used.

2.4. Protein Quantification

2.4.1. Placental Alkaline Phosphatase (PLAP) Assay

2.4.1.1. QUANTI-Blue™ colorimetric enzyme assay

The PLAP (Placental Alkaline Phosphatase) gene was used in this project as a reporter gene to measure the *AOX1* promoter expression levels in *Pichia pastoris* cultures. PLAP production and concentration was assessed using the QUANTI-Blue™ colorimetric enzyme assay (InvivoGen, San Diego, CA, USA). The assay was performed by adding 100 µl of sample/standard to 900 µl of QUANTI-Blue™ medium (QB) in spectrophotometer or 20 µl sample in 180 µl QB in a 96-wells plate reader (Safire2 microplate reader, Tecan, CH). The reaction media changed colour from pink to blue proportionally to the amount of PLAP present in the media. PLAP production was assessed qualitatively with the naked eye or quantitatively using a spectrophotometer at 620 nm (http://www.invivogen.com/quanti-blue). The reaction was carried out, for 96-well plate reader analyse, in a white, clear bottom 96-wells plate (Costar, Fisher Scientific, UK).

2.4.1.2. NovaBright™ Chemiluminescent Detection System

PLAP can also be assessed by chemiluminescence assay (NovaBright™ Alkaline Phosphatase Enzyme Reporter Gene Chemiluminescent Detection System 2.0, Catalogue no. N10577, Invitrogen). To 100 µl of sample/standard were added 200 µl of Assay buffer. Samples were incubated at 65°C for 5 minutes. 200 µl of reaction buffer were added. 120 µl of this mixture were aliquoted into a white, clear bottom 96-wells plate. Samples were read after 20 minutes incubation at room temperature by a 96-wells plate reader (Safire2 microplate reader, Tecan, CH). For the reaction white and black, clear bottom 96-wells Costar plates (Fisher Scientific, UK) were used.

2.4.1.3. PLAP assay: Preparation and calculations

Sample preparation

All samples (1 mL) taken in this work from shake flask or bioreactor cultivation have been centrifuged at 250 rpm for 2 minutes. The supernatant has been measured for PLAP activity with the assays described in the previous sections. The pellet has been resuspended in ddH_2O, spun down again and resuspended once more in ddH2O to a final volume of 1 mL. The samples have then been sonicated for 10 seconds to disrupt the cells. After that the samples were centrifuged and the supernatant was measured for PLAP activity as described in the previous sections.

Calculations

The PLAP activities found for both the crude supernatant (PLAP secretion) and the supernatant after cell disruption (non secreted PLAP) were added one to the other in order to obtain the total PLAP concentration.

2.4.2. Bradford Assay

The calibration curve was obtained with serial dilutions of BSA (bovine serum albumin) (Fermentas GMBH, UK). The amount of total protein in the supernatant was obtained by adding 50 µL of sample/standard into 950 µL Bradford reagent (Sigma Aldrich Company LTD, UK) and by reading the absorbance at 595 nm.

2.5. Transient expression of Placental Alkaline Phosphatase (PLAP) in an Immortal Mammalian Cell Line (HeLa)

Due to its cost and the amount needed, some in-house PLAP standard was produced for assay optimisation purposes. The standard was produced through a transient expression of PLAP in HeLa cells. The protein was secreted into the supernatant and was used the un-purified broth as a standard, as the assay (chemiluminescent) did not require purification.

1 mL of HeLa cells stored in liquid nitrogen were resuspended in 10 mL DMEM (L-glutamine^{+}; 10% FCS) (Sigma Aldrich Company LTD, UK) and centrifuged 5 minutes at 470 rpm to remove DMSO (Sigma Aldrich Company LTD, UK). The pellet was resuspended in 10 mL of the same medium and incubated in a 6-wells plate in serial

dilutions at 37˚C. Cells were passaged after 4 days incubation as follows: Supernatant was removed from each well and cells were washed with 0.5 mL PBS (Sigma Aldrich Company LTD, UK). 0.5 mL Trypsin EDTA (Sigma Aldrich Company LTD, UK) were added after removing PBS and the plate was incubated 5 minutes at 37˚C. After incubation cells were detached by pipetting up and down using 1 mL of DMEM medium containing 10% FCS.

Cells were then re-plated in three different dilutions and in double and incubated overnight at 37˚C. Transfection was performed using the purified pSEAP2-control (figure 2.4) vector (Clontech Laboratories, Inc.) at a final concentration of 0.1 µg/µL following the SuperFect Transfection Reagent (Qiagen, Crawley - West Sussex, UK) protocol. Samples were taken after 24 and 48 hours to assay PLAP production.

2.6. Methanol Quantification Assay

The reaction aimed to determine residual methanol (Figure 2.5) in a biological sample was performed either in a conventional spectrophotometer or in a 96-well plate reader at a wavelength of 346 nm. The original reaction was described by Badrakhan *et al.* (2004) as a mean to measure DHAA (dehydroascorbic acid) in biological samples using methanol as a reagent. However, in this study DHAA is used to quantify methanol.

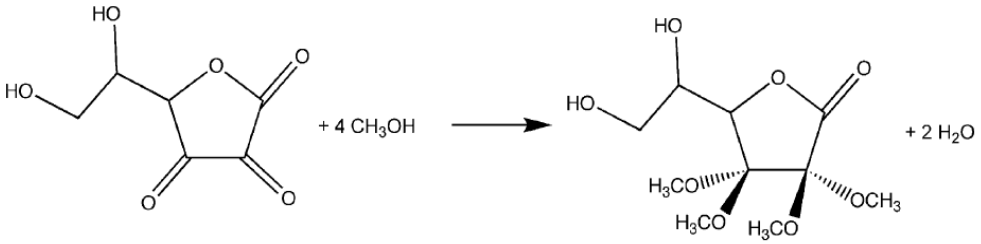


Figure 2.5: Reaction between methanol and dehydroascorbic acid (DHAA)
Reaction between methanol (CH_3OH) and DHAA (dehydro-ascorbic acid – MW = 174.108 g/mol). This reaction s performed in phosphate/citrate buffer. It yields several products, of which the main derivate is displayed above (Badrakhan *et al*., 2004).

The reaction was tested in phosphate/citrate buffer (0.4M NaH_2PO_4 / 0.125M Citric Acid Monohydrate) adjusted to pH 4.2, 6.4, 7.75 and 9.8 with NaOH (Sigma Aldrich Company LTD, UK), or with 0.5M or 1M phosphate buffer (see Chapter 2). Different reaction pH (4.2, 7.2, and 9.8) were tested in different media. DHAA (Sigma Aldrich Company LTD, UK) was used as a principal reagent as outlined in figure 2.5, and different concentrations (stock solution: 156.22 mmol/L) of this reagent were tested against different concentrations of methanol in several biological samples in presence and absence of a catalyser (15.4 mM DAM – Desferrioxamine mesylate - VWR International LTD, UK). Several ratios of Sample:DHAA:Buffer (2:2:4; 1:1:2) have also been tested. The reaction was tested at both room temperature (RT) and 37°C and readings were taken every minute for 30 minutes in spectrophotometer or 96-wells plate reader.

3. Development of Bioprocess monitoring methods

3.1. Introduction

During *Pichia pastoris* fermentation methanol accumulation rate is a key parameter for successful cultivation. In fact, methanol accumulation should be avoided in methanol limited fed-batch protocols, or should be kept constant in oxygen limited cultivations. Failure to do that will result either in higher cell death rate, or in irreproducible data (Pla *et al.*, 2006). Current methanol detection systems are either expensive (methanol probes) or laborious and time consuming (e.g. gas chromatography). Methanol probes are widely used especially in industry, and they can offer real-time data about methanol utilisation and accumulation. Among on-line measurement methods, mass-spectrophotometry is also used. However, in the late methanol induction phase high concentrations of oxygen are used, and having methanol the same molecular weight of oxygen, results are often incorrect, as they are highly dependent on oxygen concentration variations. In some cases, mass spec is impractical as in lines that connects the fermenter with this machine are very long and, in the worst cases, not heated. As a result, methanol gases will condensate along the line and mass spec reading will not be representatives (Beynon *et al.*, 1968; Litchmann & Upton, 1972).

On the other hand, methods such as gas chromatography are highly reproducible. The drawback is that cells have to be separated from culture broth before assay. Coupled with long analysis times (usually over 20 minutes, results on methanol concentrations will be available only after 30 minutes from the time the sample is taken. In addition, during fermentation, an almost dedicated mass spec is needed, and in most cases this is not possible for different reasons, such as lack of other machines or too many users of the

same instrument. As a consequence, analysis may be delayed, creating concerning about safety (Beynon *et al.,* 1968; Litchmann & Upton, 1972). Fermentation methods requiring high methanol feed rate may suffer from this delay. In this case, methanol accumulation was simply controlled by monitoring DO spikes in the fermenter (i.e. if there is not accumulation, DO-spike should happen shortly after stopping the methanol feed pump). Therefore, there was the need to create a new and fast way to monitor methanol concentrations inside the vessel.

In order to correctly test PLAP volumetric yield and productivity levels an assay testing and optimisation was needed. Two different assay (chemiluminescent and colorimetric) were compared and optimised. First a chemiluminescent assay is described and analysed, whereas the colorimetric assay is outlined at the end.

3.2. At-line DHAA-based assay for Methanol levels

Methanol is used in the food industry to measure the concentration of the Vitamin C derivate dehydro-ascorbic acid (DHAA) in food. In this 346 nm spectrophotometrical assay methanol is used in molar excess to detect DHAA in biological samples (Badrakhan *et al.*, 2004). This Section was aimed to determine if the reverse reaction, where DHAA is used to detect methanol, can be applied to measure methanol in bioreactors.

As showed in Chapter 2, many different concentrations of methanol and DHAA have been tested at different pH values in both Tecan (96 wells) and spectrophotometer (1 cm cuvette). The first tests were performed by spectrophotometer and the preliminary

results were encouraging (Figures 3.1 and 3.2). However, after these tests were repeated the reproducibility was high for methanol concentrations above 10 g/L (Figure 3.3) and poor for lower concentrations, which showed high background noise.

After few initial experiments in a spectrophotometer, Tecan tests were started as multiple samples could be analysed at the same time and with different conditions. As resolution (qualitative measure of the angular separation of data trends that are close together), for low methanol concentrations was poor (Figure 3.4), samples composition was changed in order to reduce the amount of DHAA used and to reach the same path-length (1 cm) that can then be used once the test is optimised for rapid assay in spectrophotometer (less than 10 minutes). Ideally, the objective was to increase the assay resolution for concentrations of methanol lower than 3.0% (v/v), as that would be the upper limit *Pichia pastoris* cells could tolerate.

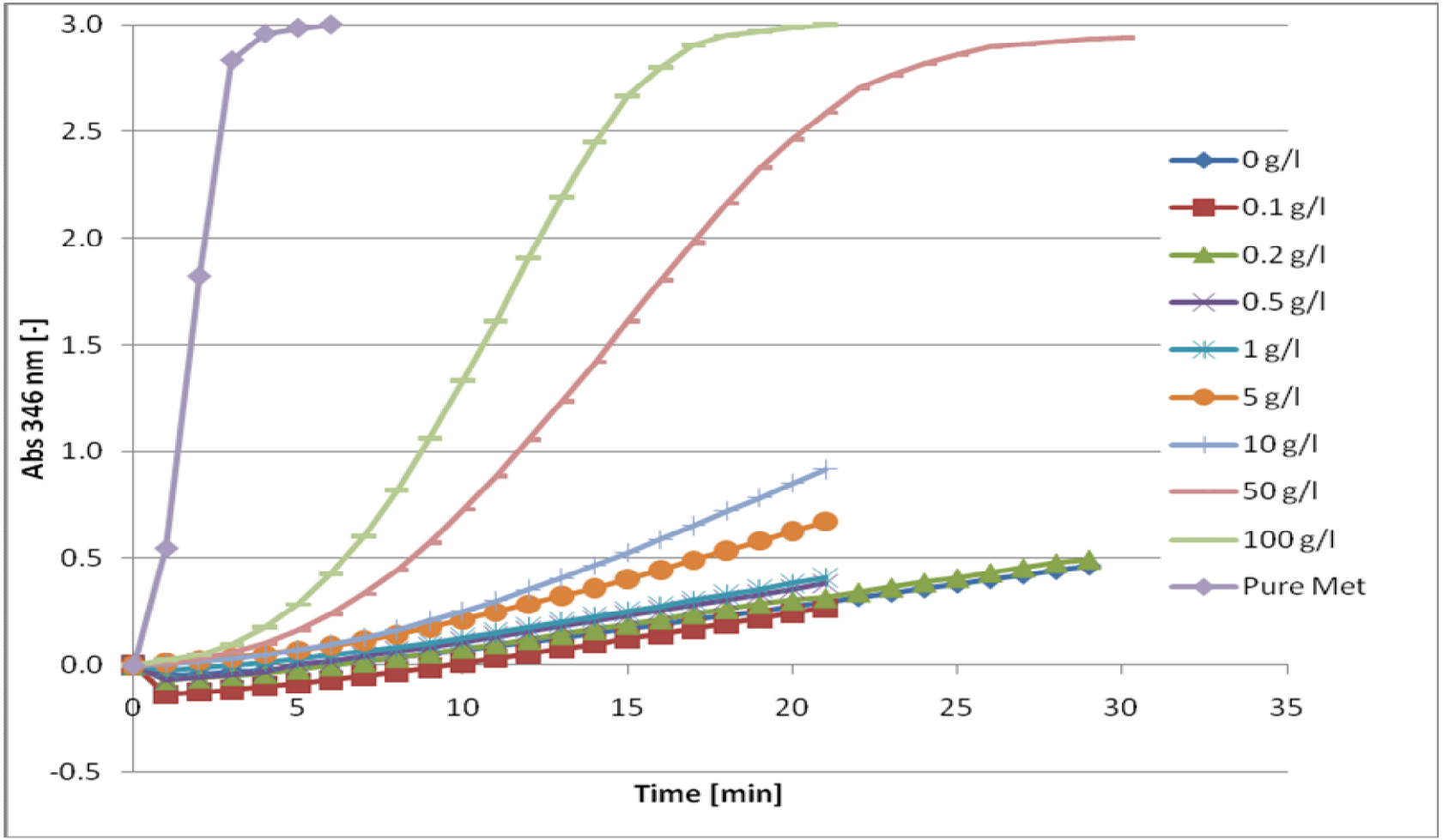


Figure 3.1: Spectrophotometrical analyse of different methanol concentrations

This graph shows experimental data obtained by spectrophotometer for the quantification of methanol by dehydroascorbic acid. Sample composition: Phosphate/citrate buffer (0.4 ml), pH 7.75, 78.11 mM DHAA (0.2 ml), different methanol concentrations (0.4 ml). High resolution for high methanol concentrations (equilibrium reached). Low resolution for lower concentrations and high background noise.

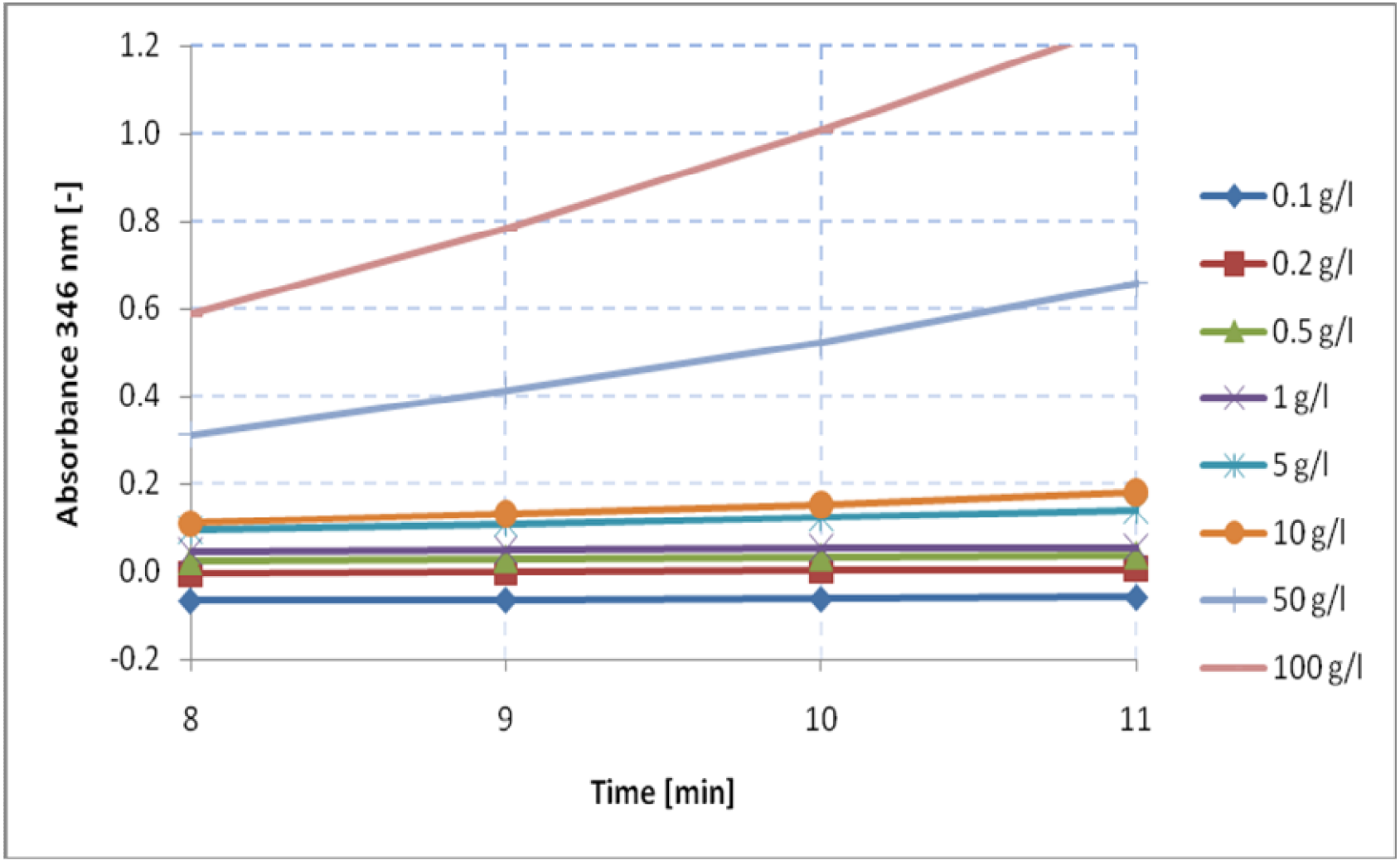


Figure 3.2: Resolution analyse of different methanol concentrations by spectrophotometer

This graph shows experimental data obtained by spectrophotometer for the quantification of methanol by dehydroascorbic acid. Sample composition: Phosphate/citrate buffer (0.4 ml), pH 7.75, 39.1 mM DHAA (0.2 ml), different methanol concentrations (0.4 ml). Best resolution after 9 minutes of reaction. Resolution, in this case, is the qualitative measure of the angular separation of data trends that are close together.

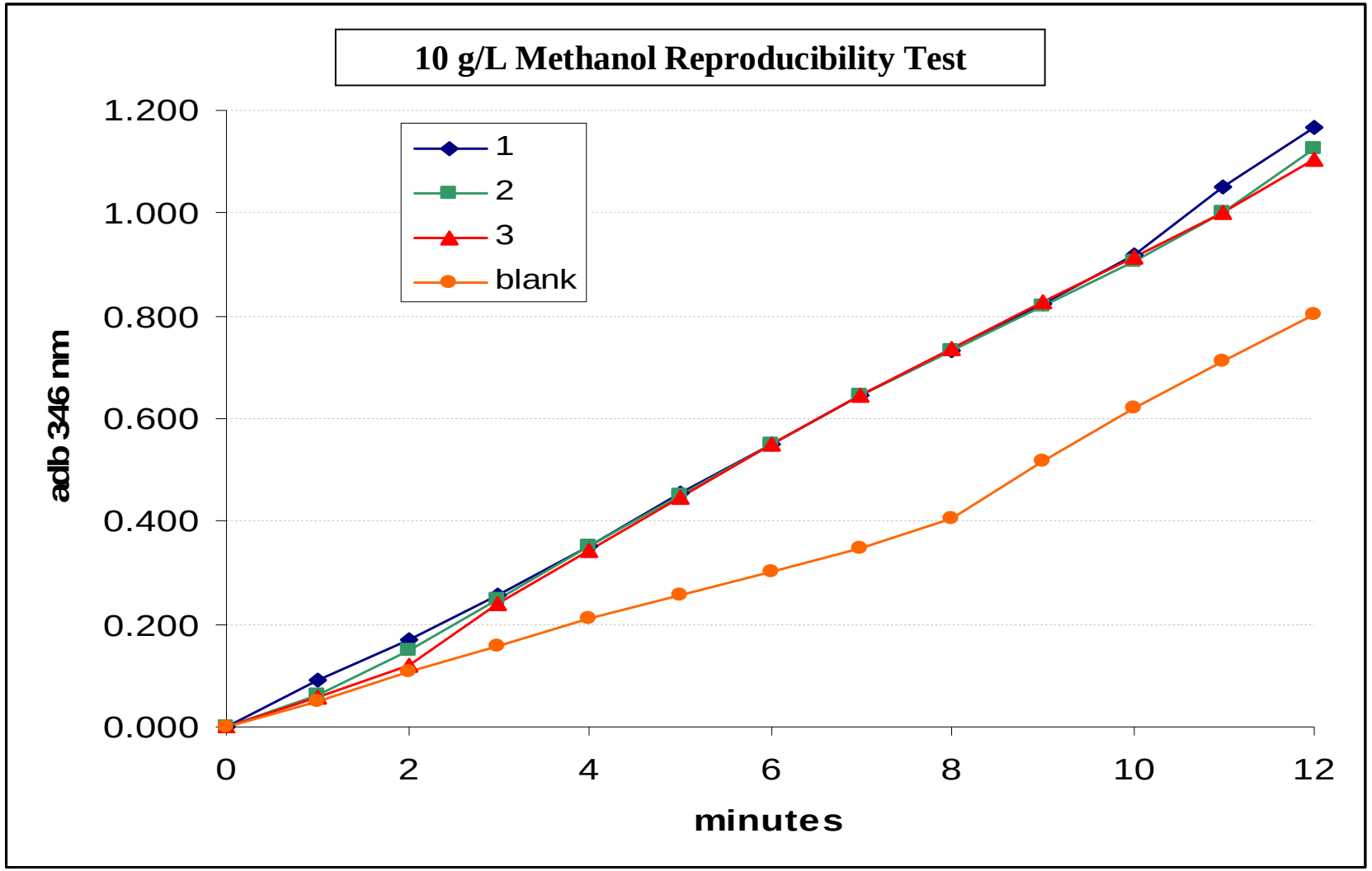


Figure 3.3: Spectrophotometrical reproducibility assay for the reaction between methanol and DHAA
This graph shows experimental data obtained by spectrophotometer for the quantification of methanol by dehydroascorbic acid. Sample composition: Phosphate/citrate buffer (0.4 ml), pH 9.4, 78.11 mM DHAA (0.2 ml), 10 g/L methanol (0.4 ml). High reproducibility between 3 and 10 minutes reaction time.

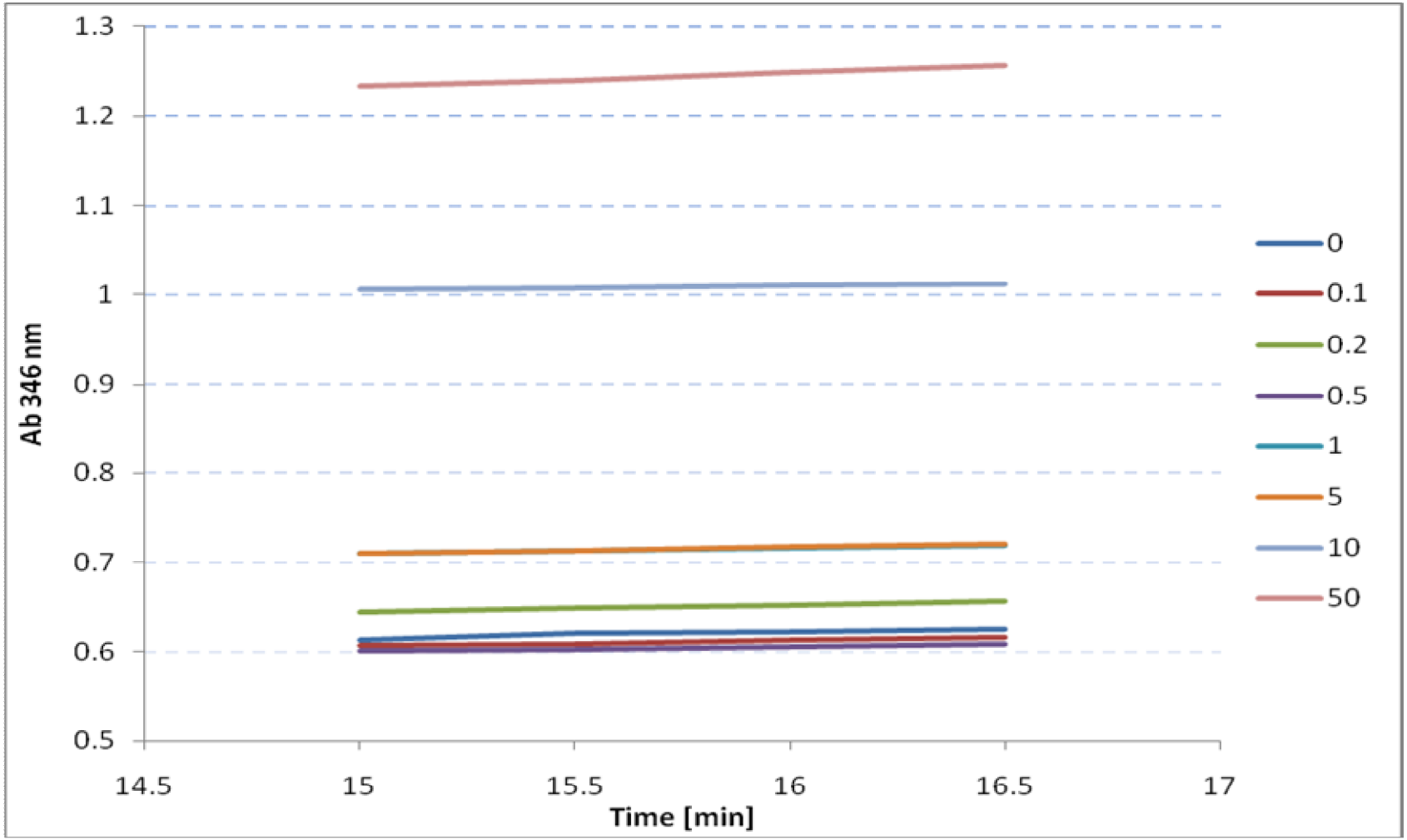


Figure 3.4: Analyse of different methanol concentrations in a 96-wells plate reader

This graph shows experimental data obtained by Tecan for the quantification of methanol by dehydroascorbic acid. Sample composition: 1 M Phosphate buffer (120 µl), pH 7.2, 9.8 mM DHAA (60 µl), different methanol concentrations from 0 to 50 g/L (120 µl). High resolution for high methanol concentrations. Low resolution for lower concentrations and high background noise.

From the results, it was noticed that low DHAA concentrations performed better (Figure 3.5) than high concentrations for two main reasons. First, the Tecan reads absorbance in vertical (spectrophotometer in horizontal). Since high concentrations of DHAA tend to precipitate, reading in the Tecan would be affected more than in a spectrophotometer. The second reason is that the original reaction is performed with high excess of methanol to detect low amounts of DHAA. Consequently, low amounts of DHAA are limiting for high methanol concentrations, leading to faster DHAA saturation and DHAA-Methanol equilibrium (Figure 3.1). As a result, the concentration of DHAA was set to a theoretical mid-point compromise in which its concentration was not low enough to have an instantaneous reaction and not high enough to precipitate. Reaction conditions to measure both low and high amounts of methanol were found, even if low methanol concentrations tend to be unstable.

By changing the reaction buffer the reaction times could be further optimised. As the final objective was to test methanol in biological samples, methanol was tested in water, as well as in different media (Figure 3.6). Results showed that there was no influence from the media used upon the reaction itself, as the absorbance readings varied only in relation of the absorbance of the media (blank) at 346 nm. However, fluctuations for low methanol concentrations once more showed unsuitability of this assay to detect methanol titres below 4 g/L (Figure 3.7).

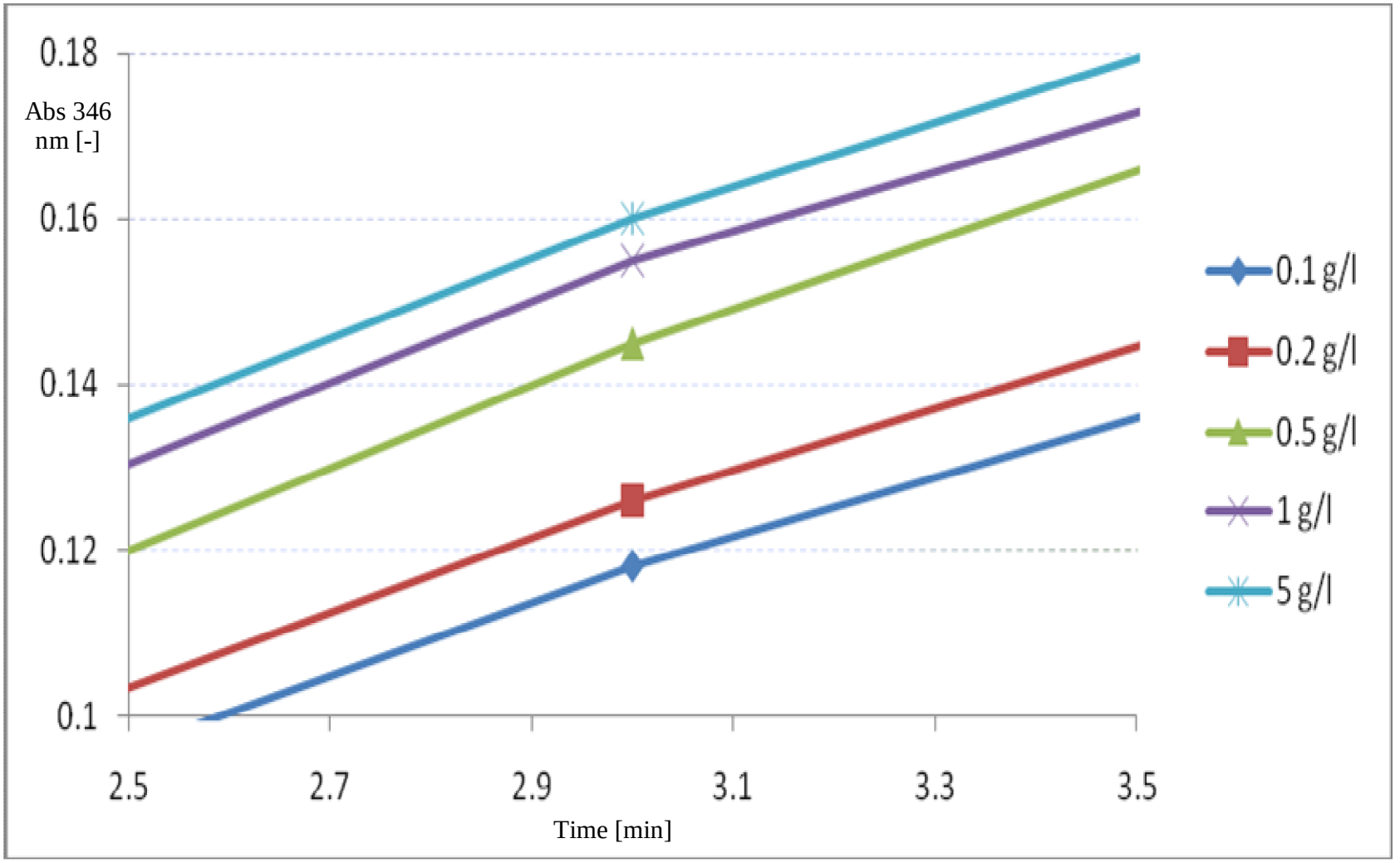


Figure 3.5: Low methanol concentration screening in Tecan

This graph shows experimental data obtained by Tecan for the quantification of methanol by dehydroascorbic acid. Sample composition: 1 M Phosphate buffer (120 µl), pH 9.8, 3.91 mM DHAA (60 µl), different methanol concentrations from 0 to 50 g/L (120 µl). High resolution for low methanol concentrations.

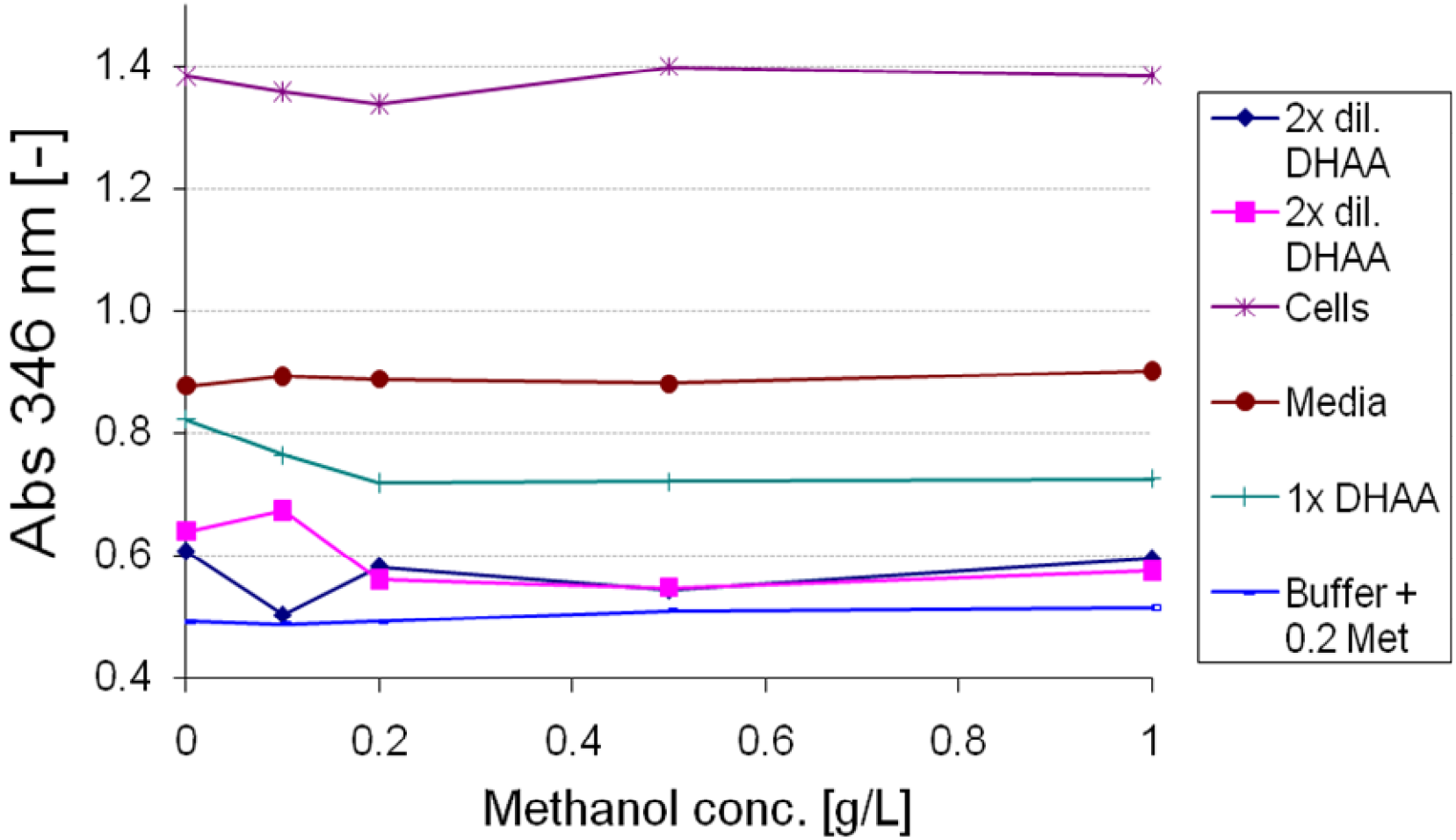


Figure 3.6: Media influence for the reaction between methanol and DHAA at methanol concentrations below 1%
This graph shows experimental data obtained by Tecan for the quantification of methanol by dehydroascorbic acid. Sample composition: Phosphate/citrate buffer (80 µl), pH 7.75, 39.1 mM DHAA (40 µl), water or media (80 µl). Media tested: 2x dil. DHAA: 39.1 mM DHAA; Cells: Filtered *Pichia pastoris* Fc strain from Merck culture in YPD media; Media: YPD broth; 1x DHAA: 78.11 mM DHAA; Buffer + 0.2 Met: Buffer containing 0.2 g/L methanol. Readings taken after 9 minutes reaction. Media did not influence reaction, but only the absorbance absolute value.

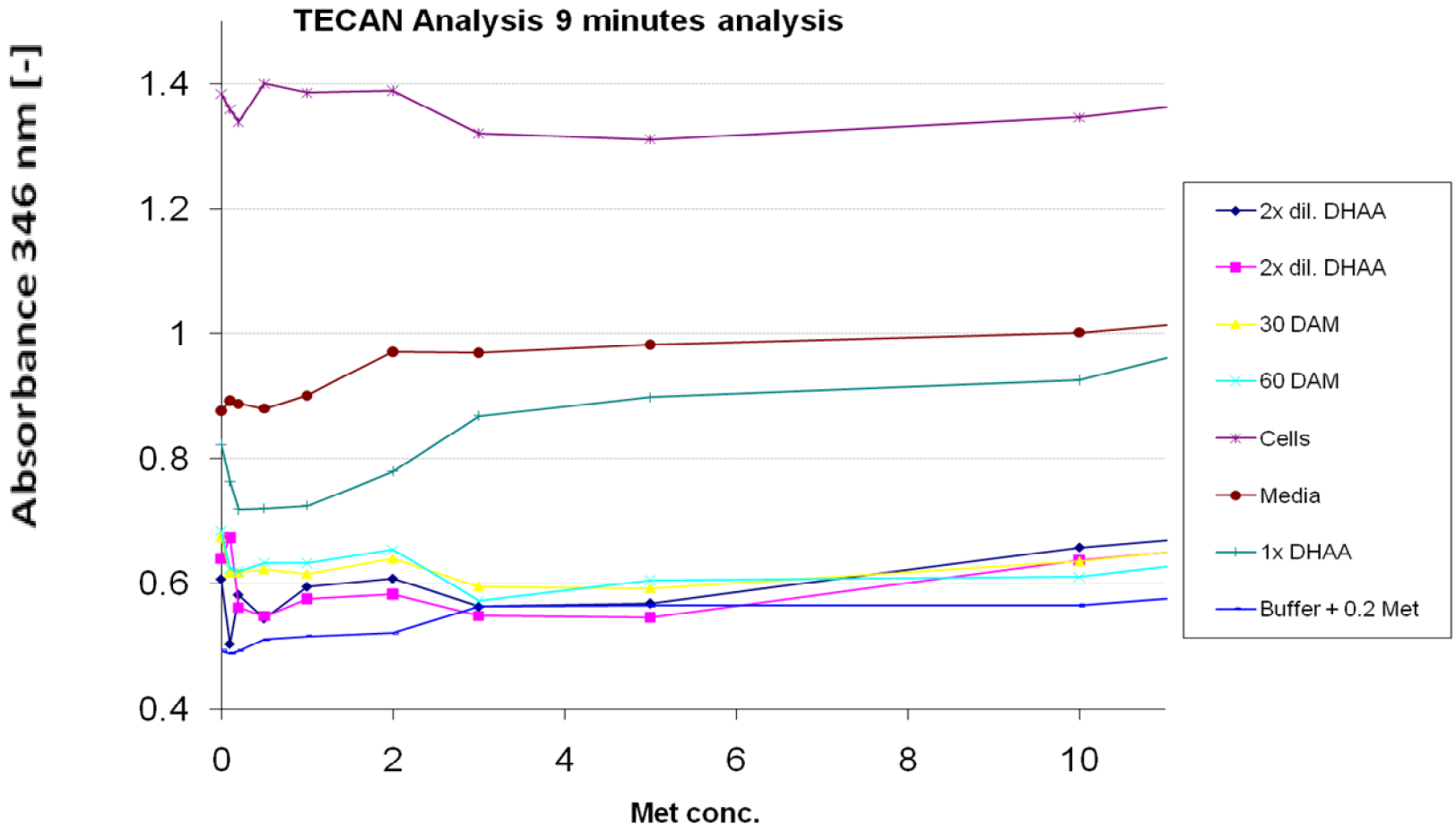


Figure 3.7: Media influence for the reaction between methanol and DHAA at methanol concentrations between 0 and 10%
This graph shows experimental data obtained by Tecan for the quantification of methanol by dehydroascorbic acid. Sample composition: Phosphate/citrate buffer (80 µl), pH 6.4, 39.1 mM DHAA (40 µl), water or media (80 µl). Media tested: 2x dil. DHAA: 39.1 mM DHAA; Cells: Filtered *Pichia pastoris* Fc strain from Merck culture in YPD media; Media: YPD broth; 1x DHAA: 78.11 mM DHAA; Buffer + 0.2 Met: Buffer containing 0.2 g/L methanol. Readings taken after 9 minutes reaction. Media did not influence reaction, but only the absorbance absolute value. However, low methanol concentrations increased variation level. Therefore, this conditions were not suitable to detect methanol concentrations below 4 g/L.

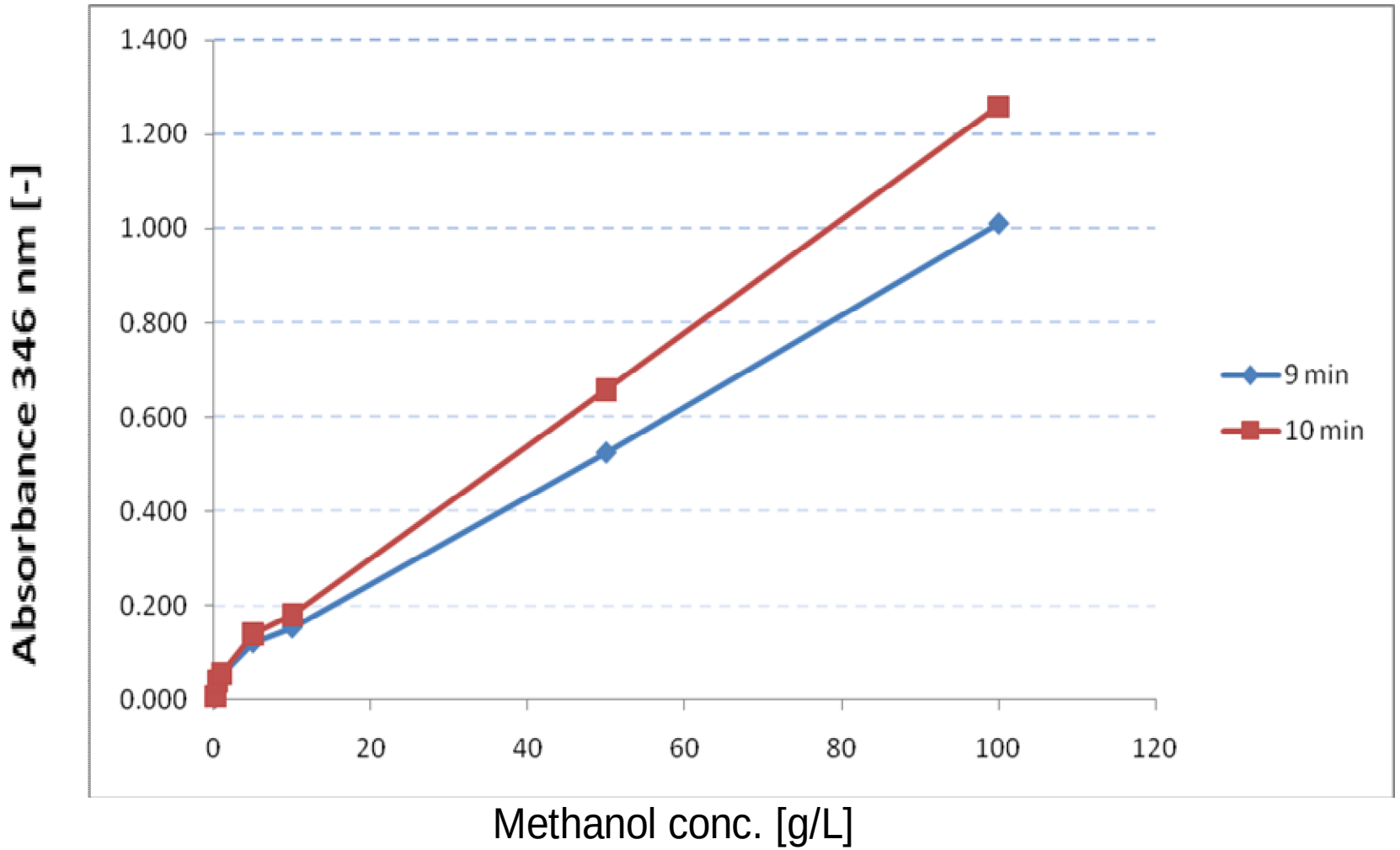


Figure 3.8: 9 versus 10 minutes reaction times at different methanol concentrations
This graph shows experimental data obtained by Tecan for the quantification of methanol by dehydroascorbic acid. Sample composition: 1M Phosphate buffer (0.4 ml), pH 9.4, 39.1 mM DHAA (0.2 ml), different methanol concentrations (0.4 ml).

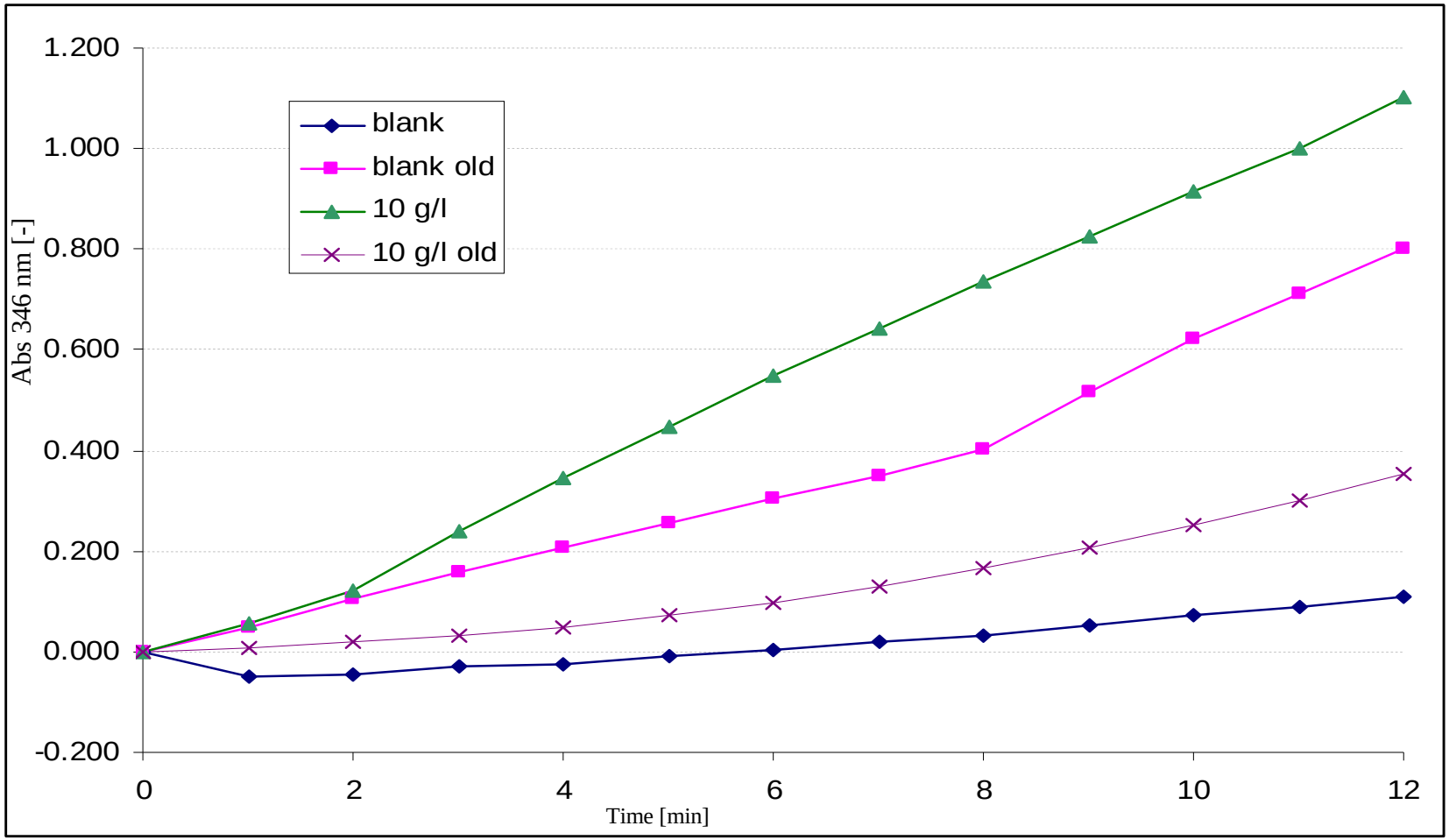


Figure 3.9: Reproducibility assay for the reaction between methanol and DHAA in tecan
This graph shows experimental data obtained by Tecan for the quantification of methanol by dehydroascorbic acid. Sample composition: Phosphate buffer (0.4 ml), pH 9.4, 39.1 mM DHAA (0.2 ml), 10 g/L methanol (0.4 ml). Poor reproducibility for both blank and 10 g/L methanol.

Finally, the reaction time that offered the best reproducibility was between 9 and 10 minutes, even though low methanol concentrations were still not fully detectable (Figure 3.8). Further development of the assay would involve a screening of multiple buffers to individuate one that could help reaching higher resolution for low methanol concentrations. However, to date poor reproducibility was recorded in Tecan (Figure 3.9), even for concentrations of methanol higher than 10 g/L.

3.3. Off-line Assay Comparison for PLAP production

3.3.1. Chemiluminescent assay for alkaline phosphatase activity

In order to quantify the amount of PLAP in biological samples a commercially available assay that quantifies the protein activity via chemiluminescence in a 96-wells plate reader (see material and methods section) was used. However, after performing a standard curve (Figure 3.10) an unfavourable linear correlation of the measures was noticed. In fact a plateau for the two most concentrated samples was recorded, and therefore, the most concentrated standard measure was removed (Figure 3.11). Also in this case linear correlation of points was not ideal. The measure was repeated several times using both a commercial PLAP standard (included in the kit) and an in-house made one. For all runs, results were discouraging and a more in-depth view of the 96-wells plate was needed. First, the plate was read with no samples, and the reading was as expected, with only minor fluctuations from well to well (Table 3.1). Then, another plate was read with all 96 wells filled with the reagent and water as sample. The reaction was performed following the protocol treating water as normal samples. Here again fluctuations were a little bigger (possibly because of minor volume errors in wells due to

pipetting), but still within an acceptable range (+/- 21 [-]) due to the magnitude of a chemiluminescent reading (several hundred to several thousand units).

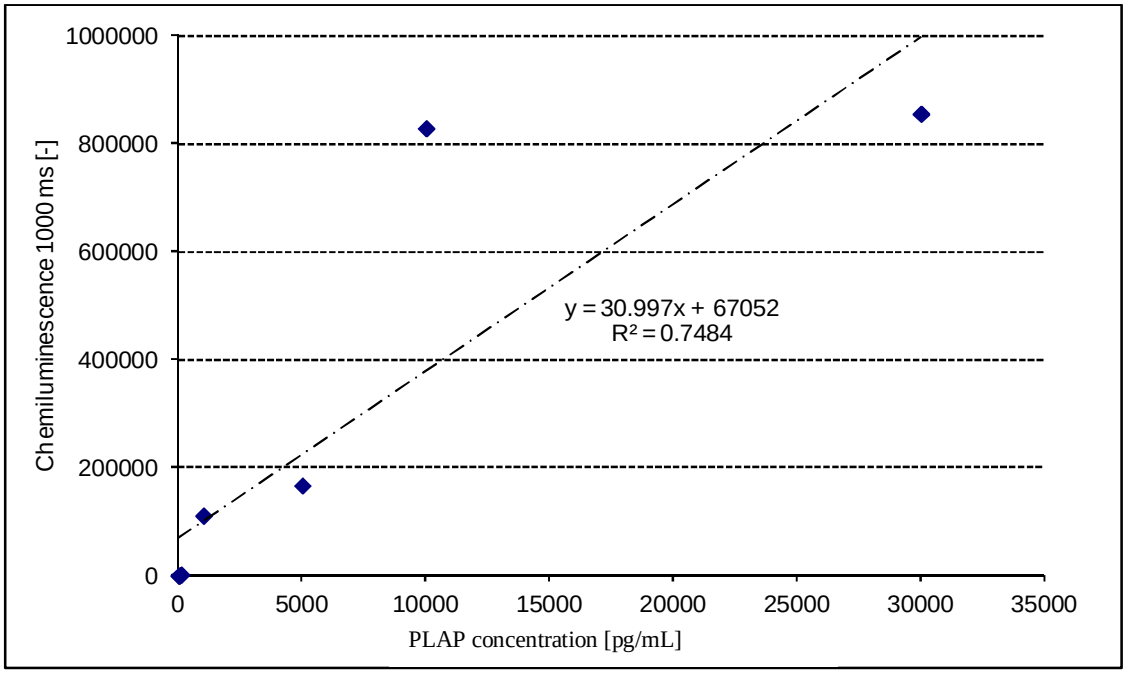


Figure 3.10: PLAP calibration curve using a chemiluminescent assay
Poor linear correlation was recorded, with an obvious plateau at high PLAP concentration.

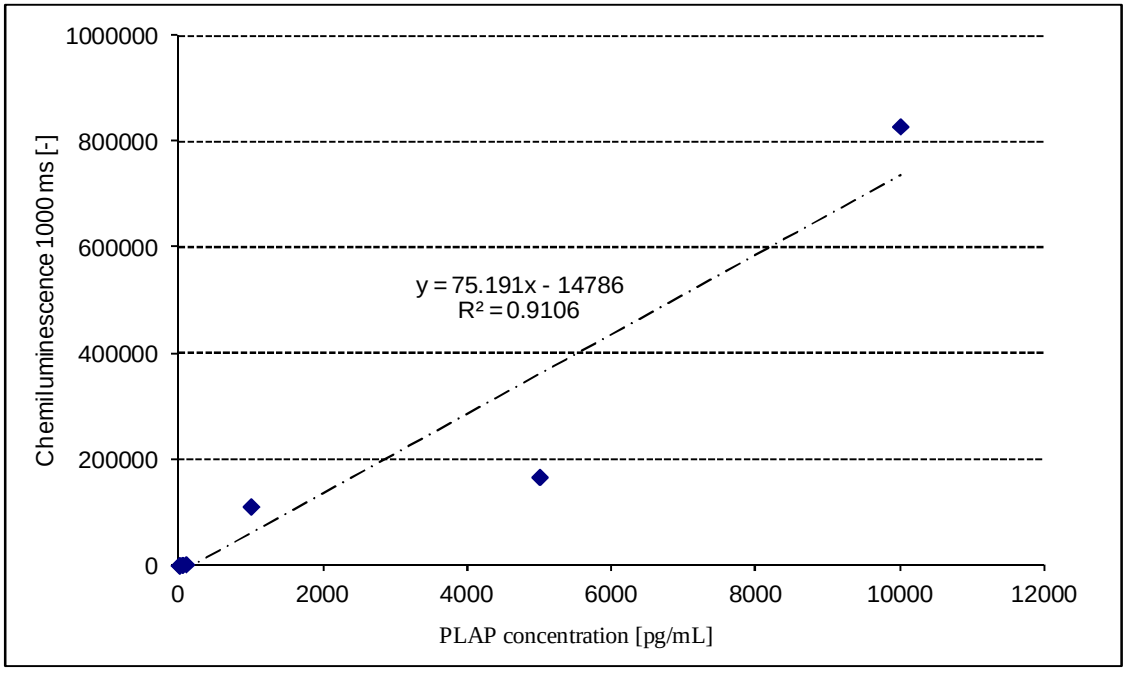
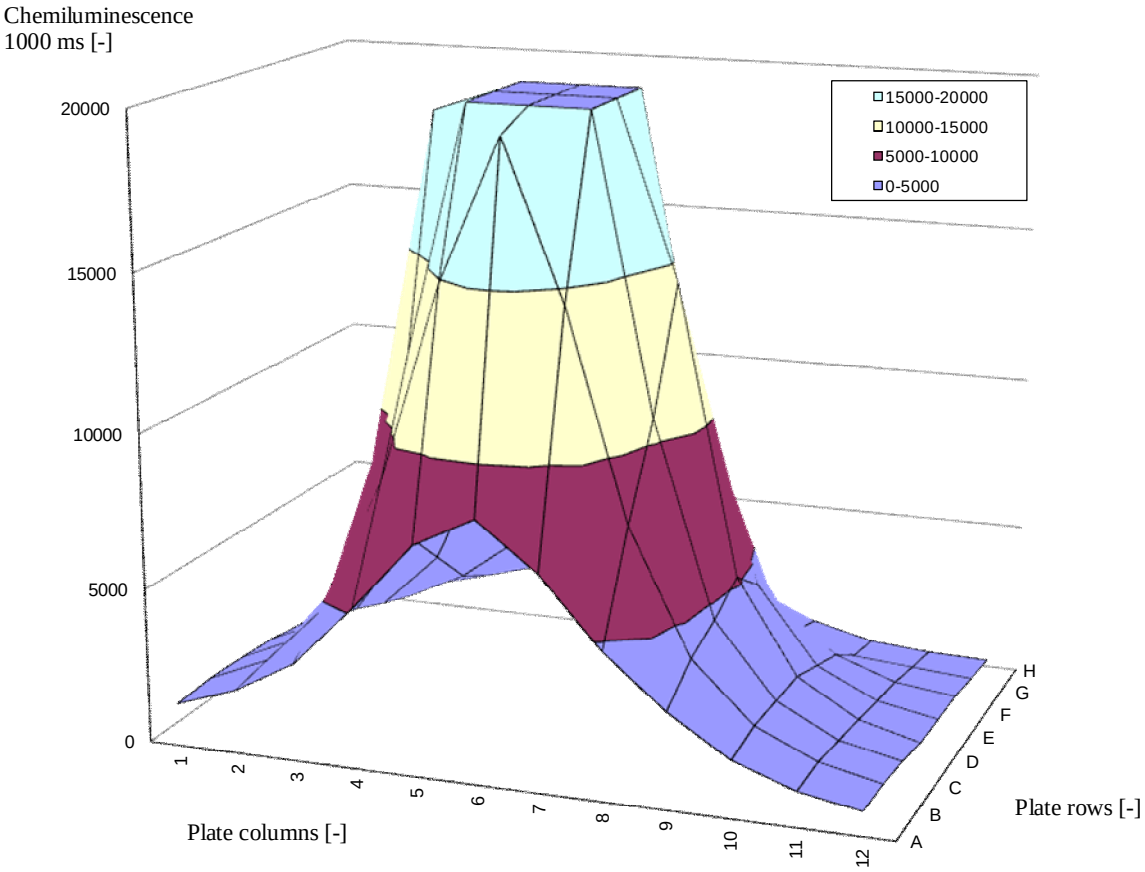


Figure 3.11: Corrected PLAP calibration curve using a chemiluminescent assay
A non ideal linear correlation was recorded.

Table 3.1: Empty plate chemiluminescent reading
Empty 96-wells plate reading taken in 96-wells plate reader with chemiluminescent method – 1000 ms. Very low level of noise was recorded, consistent in the whole plate.

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1	11	2	1	-1	2	4	1	4	2	5	1
B	1	-1	0	-1	0	1	0	-2	6	1	3	3
C	0	-1	-1	4	2	2	1	3	1	-1	1	3
D	0	1	2	3	0	0	5	3	6	1	0	3
E	2	2	2	2	1	0	3	6	3	0	2	-1
F	3	-2	1	5	2	2	1	0	2	1	-2	1
G	0	0	-2	3	-1	5	5	1	1	6	-1	1
H	1	0	0	2	0	-1	1	2	1	7	1	1

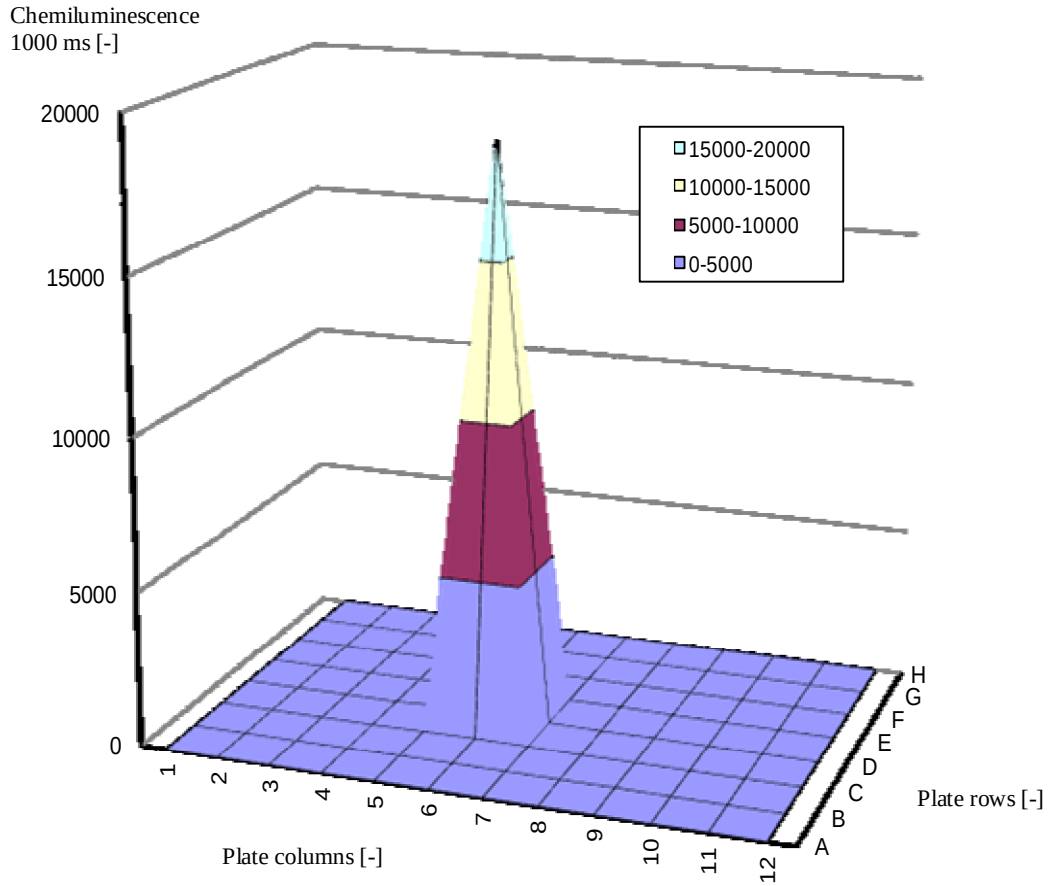
The next step was to put a highly concentrated sample in the middle of the plate and to take the chemiluminescent reading as from protocol. The reading was quite high in adjacent wells across the plate (Figure 3.12). It was clear that the errors in the calibration curve were related to this phenomenon.



< >	1	2	3	4	5	6	7	8	9	10	11	12
A	1373	2066	3158	5171	7375	8388	6905	4729	3095	1843	1140	785
B	1475	2304	4262	8515	14875	19461	14525	7966	4008	2124	1231	793
C	1435	2305	4898	12095	33635	61049	30552	10706	4515	2142	1113	690
D	1356	2234	5771	19320	157690	23280000	66416	14435	5212	2177	999	484
E	1175	1916	4624	14878	96367	165980	31011	10125	4273	1791	842	464
F	943	1417	3285	7517	15241	18084	11788	5956	2766	1420	717	469
G	760	985	1954	3409	4966	5667	4604	2848	1346	645	482	326
H	591	759	1290	1843	2309	2276	1979	1507	935	442	275	218

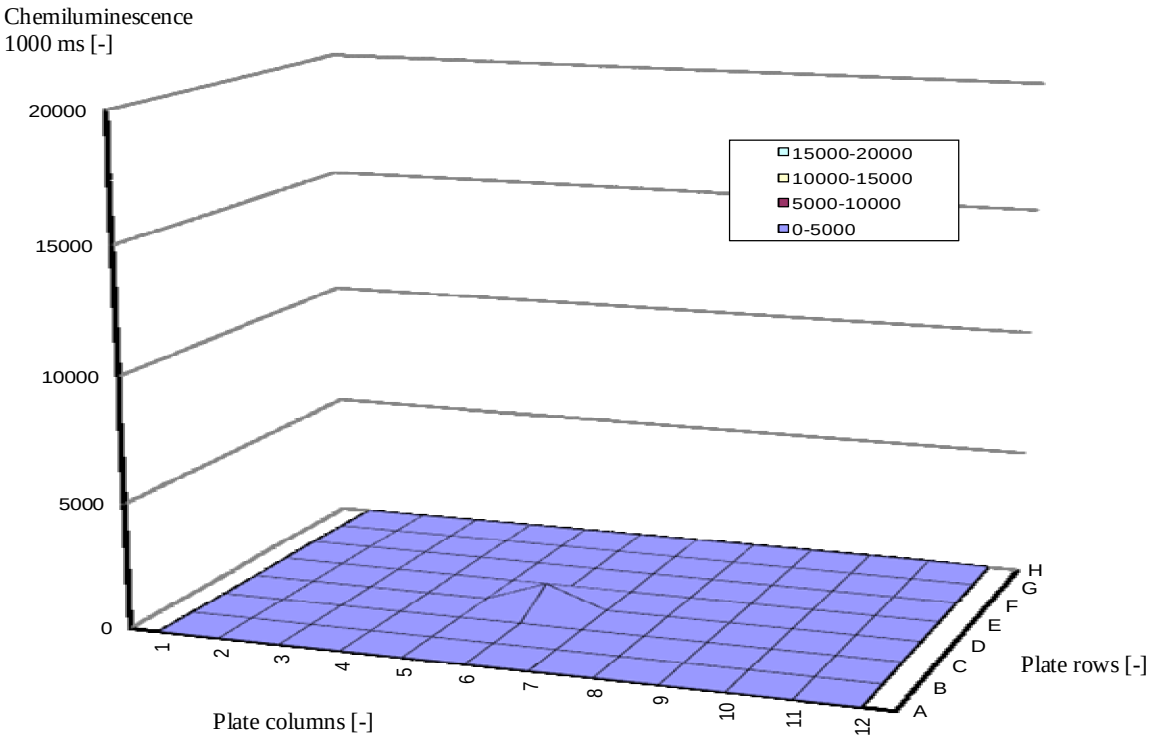
Figure 3.12: Single well chemiluminescence interference
PLAP chemiluminescence reading of a single well (highlighted in blue) in the middle of a white, clear bottom 96-well plate (Costar, Fisher Scientific, UK). The effect of the emitted light was visible in the whole plate.

The following step in the assay optimisation was to dilute the sample (Figure 3.12) used in precedence 100- (Figure 3.13) and 1000-folds (Figure 3.14). Here the effect of cross-wells contamination is noticeably reduced, especially for a 1000-fold dilution, which, however, may reduce resolution for low PLAP concentration samples. Similarly, the assay was performed using different standard concentrations (highlighted in yellow) in a black 96-wells plate (Figure 3.15) instead of the suggested white one. The rationale was that black colour should absorb light, while white plates will reflect light. In effect, a noticeable reduction in chemiluminescence magnitude of undiluted samples was recorded in comparison to the white plate. Also the effect of cross-wells contamination was reduced, but not completely removed. After this tests a better calibration curve (Figure 3.16) was obtained, but still poor reproducibility was found. In fact, estimated protein concentrations for *P. pastoris* GS116 hPLAP Mut^{+} and Mut^{S} were between 0.007 and 0.025 μg/L and between 0.009 and 0.086 μg/L, respectively. As a consequence, was decided to test another assay (next section) to quantify the target protein (PLAP). For the same reason (poor data reproducibility), chemiluminescence result values in this section have not been converted into PLAP concentration values. However, a 4 to 6 wells per plate method was used to generate preliminary data estimation for *P. pastoris* culture broth.



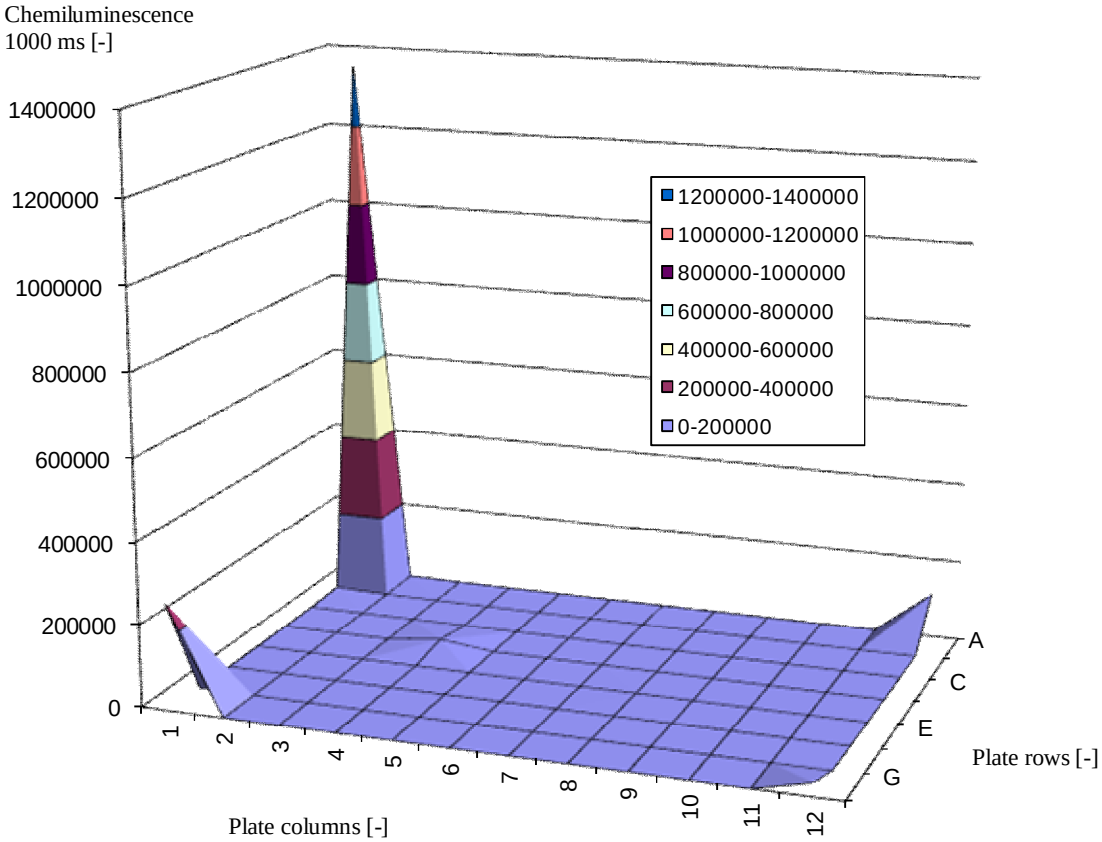
< >	1	2	3	4	5	6	7	8	9	10	11	12
A	3	5	11	5	14	7	1	7	6	3	10	1
B	3	6	3	9	21	22	16	12	18	4	5	2
C	3	5	4	7	24	41	23	6	3	1	4	5
D	5	2	12	12	90	18727	36	8	20	3	5	0
E	3	3	7	15	59	80	29	39	13	3	5	4
F	4	4	3	7	10	8	7	4	2	4	3	3
G	7	6	6	6	7	5	5	6	3	6	3	5
H	4	8	4	5	4	5	3	4	6	5	4	2

Figure 3.13: 100x diluted single well chemiluminescence interference
PLAP chemiluminescence reading of a single well (100x dilution – highlighted in blue) in the middle of a white, clear bottom 96-well plate (Costar, Fisher Scientific, UK). The effect of the emitted light for cross-wells contamination was reduced compared to an undiluted sample.



< >	1	2	3	4	5	6	7	8	9	10	11	12
A	3	-1	3	5	-1	2	1	5	2	2	2	0
B	0	6	-2	3	4	5	8	3	-2	1	2	0
C	-2	2	5	1	3	10	0	0	3	0	2	2
D	0	1	1	0	11	866	1	2	-1	5	6	0
E	3	2	-1	4	5	4	-1	8	6	3	2	3
F	4	5	3	6	3	1	3	2	4	6	2	1
G	-1	2	4	4	6	3	2	0	2	1	2	1
H	3	0	5	4	3	5	5	1	1	3	8	-1

Figure 3.14: 1000x diluted single well chemiluminescence interference
PLAP chemiluminescence reading of a single well (1000x dilution – highlighted in blue) in the middle of a white, clear bottom 96-well plate (Costar, Fisher Scientific, UK). The effect of the emitted light for cross-wells contamination was removed.



	1	2	3	4	5	6	7	8	9	10	11	12
A	1350300	2806	726	293	130	64	47	38	26	72	454	108020
B	5074	1372	540	225	104	55	28	26	39	70	290	444
C	796	513	310	185	91	45	27	24	26	42	77	53
D	306	260	217	15570	81	55	25	26	17	30	22	32
E	191	150	146	151	42	29	34	781	24	33	29	5
F	232	146	111	70	43	29	28	21	17	20	21	21
G	402	271	108	72	23	20	18	16	15	15	35	77
H	261190	506	121	59	30	24	11	9	19	19	134	33299

Figure 3.15: Black plate chemiluminescence interference
PLAP chemiluminescence reading of several PLAP standards (highlighted in yellow) in a black, clear bottom 96-well plate (Costar, Fisher Scientific, UK). The effect of the emitted light from the most concentrated samples was visible in the adjacent wells (especially well A1, which strongly influences wells A2, A3, B1, B2, and C1), but reduced in comparison to white 96-wells plates.

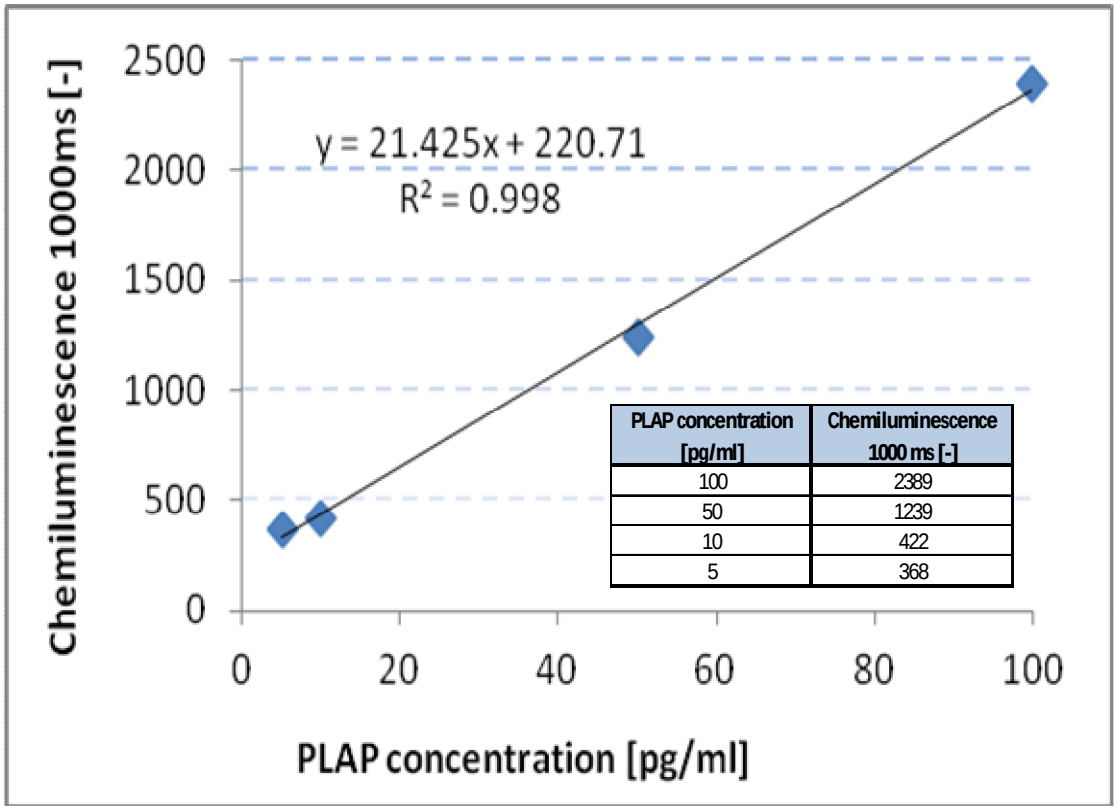


Figure 3.16: PLAP chemiluminescent assay calibration curve in black plate
Calibration curve obtained with 96-wells black, clear bottom plate (Costar, Fisher Scientific, UK). A high linear correlation was recorded.

Some in-house PLAP standard was created in HeLa cells. However, due to the high variance recorded with the chemiluminescent assay the quantification of the created standard was not possible. In fact, the amount of protein in the supernatant was estimated to be about 3.38 ± 0.98 mg/L (n=7), where the standard deviation showed an high level of error associated to the measure. As a consequence, and since the colorimetric assay was not yet available while this transient expression was performed, a commercial standard was used for colorimetric quantification. Nevertheless, the PLAP-containing supernatant generated from this experiment was used for qualitative studies, such as reproducibility and well-to-well interference tests, during assay optimisation.

3.3.2. Colorimetric assay for alkaline phosphatase activity

As discussed in the previous section, after a long unsuccessful assay optimisation study, a different assay tested. In this case was chosen a colorimetric assay (Chang *et al.*, 2009), that could also be performed in a 96-wells plate. The drawback of this assay was that low concentration samples required a long reaction time (up to 5 hours) at 37°C, and therefore the protein might be subject to degradation or proteases activity in biological samples not previously purified. However, after a few preliminary studies for reproducibility (also using proteases inhibitors cocktails) a high reproducibility was recorded and the effect of degradation of the protein was found to be negligible. Due to the long time of reaction required, different standard curves related to protein concentration (Figure 3.17) were created. In fact, very concentrated samples required 1 hour or less reaction time, whereas more diluted sample required up to 5 hours or more reaction time in order to increase resolution between samples (Figure 3.18). As a general rule, where possible the 2 hours calibration curve was used, as it was the one with the highest reproducibility. In the event of too concentrate samples, these were diluted to meet the reading range of this curve. On the other hand, with more diluted other calibration curves (3, 4 or 5 hours reaction time) had to be used depending on the actual concentration. In this case, all samples were diluted to fit the chosen calibration curve in order to use the same curve for all samples in a batch (i.e. for a whole fermentation). Standard curves were re-plotted at regular intervals, as they were always quite reproducible, and therefore there was not the need of preparing a standard curve for every reading. For biological samples a blank was required for every sample in order to remove the error generated by media colour or biological debris light absorbance (Figure 3.19).

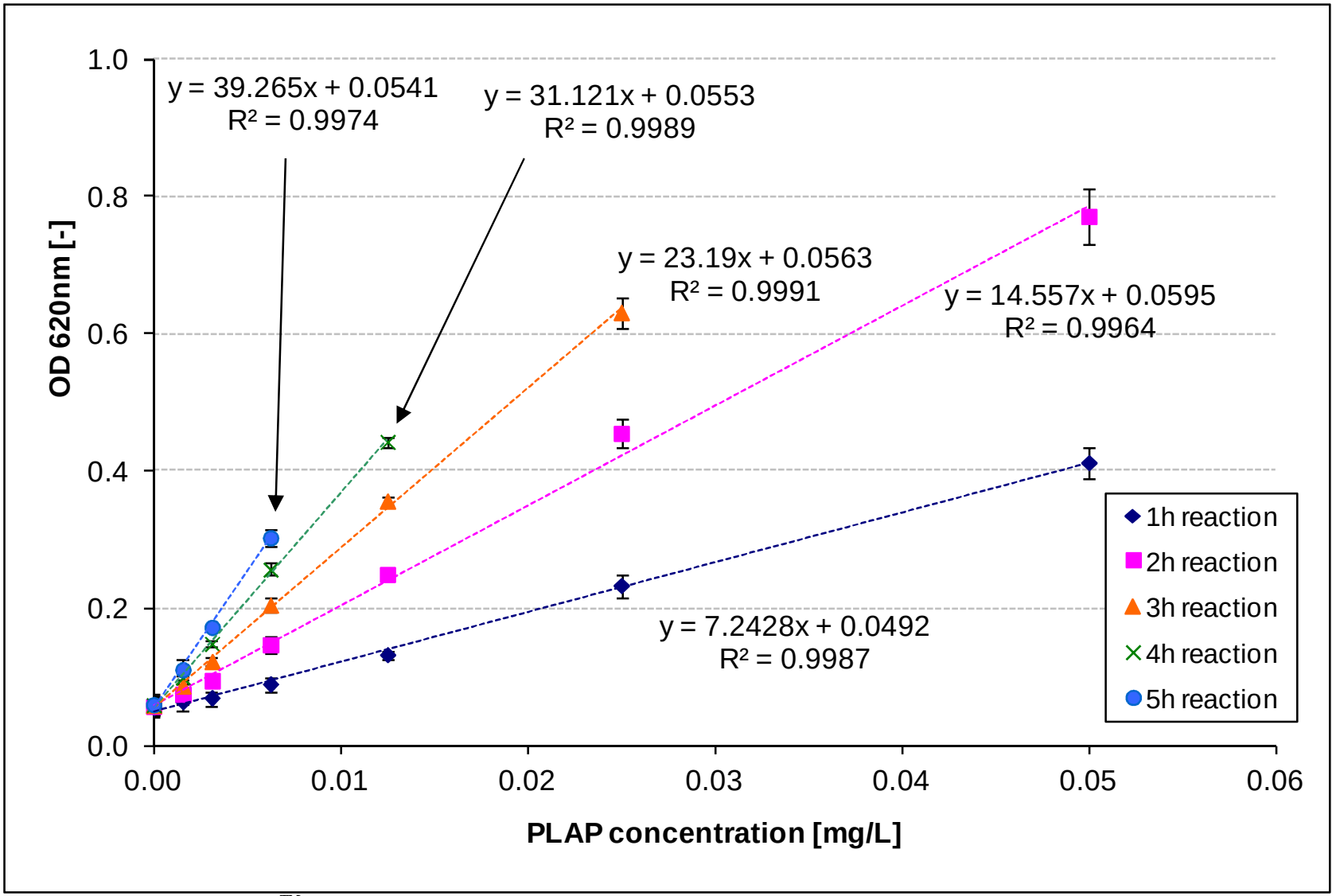


Figure 3.17: PLAP Quanti-blue™ colorimetric assay standard curves
Reading were taken from a 96-wells plate reader after every hour of reaction for 5 hours. Five standard curves were therefore generated.

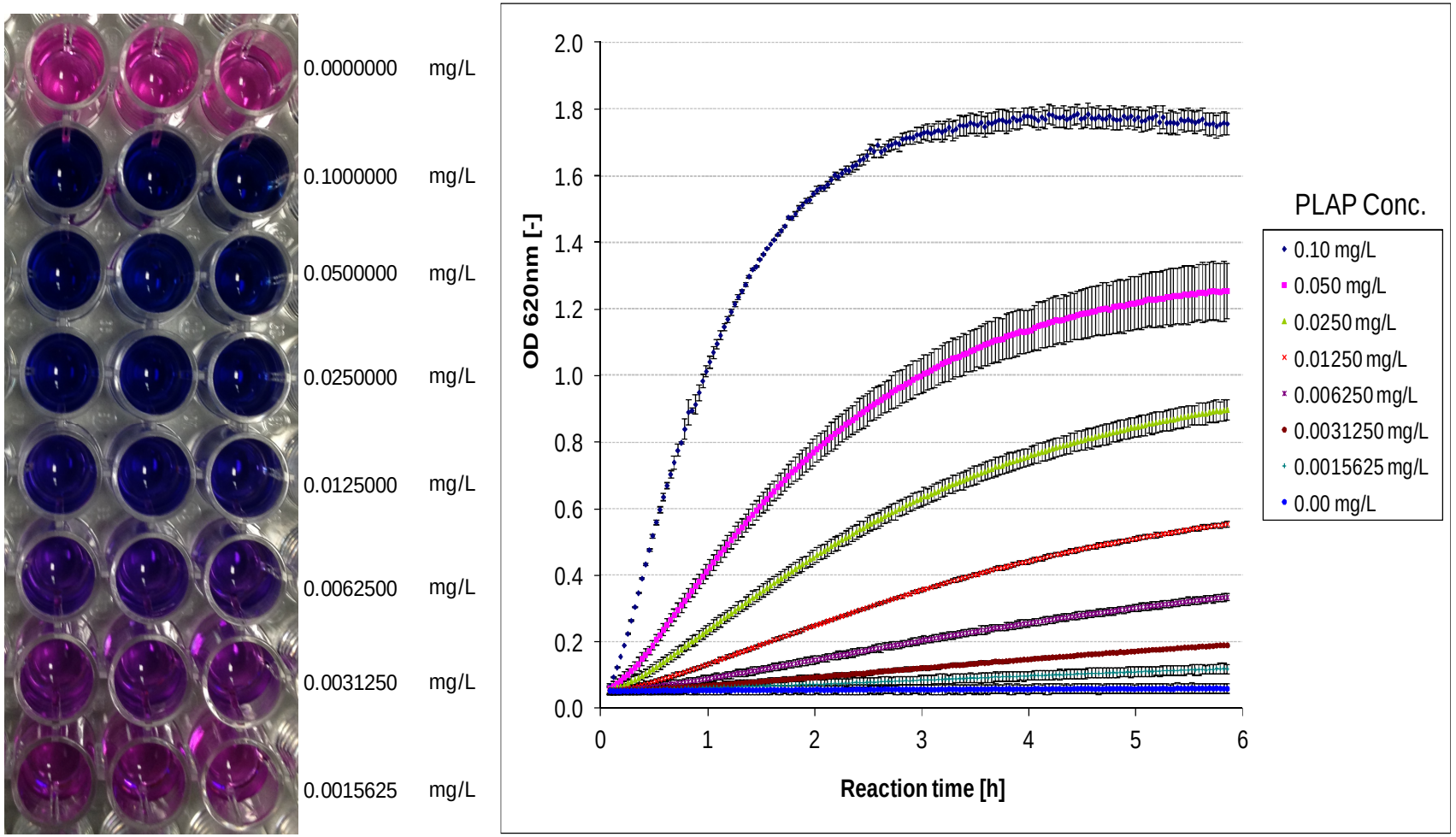


Figure 3.18: PLAP Quanti-blue™ colorimetric assay standard readings
Readings were taken from a 96-wells plate reader for 6 hours. A standard curve was generated from this readings for every standard concentration. Values were then extrapolated every hour to generate 5 standard curves (PLAP concentration vs. OD 620 nm). The picture on the left showed how sample appeared after the reaction, with pink being the lower concentration and blue the highest.

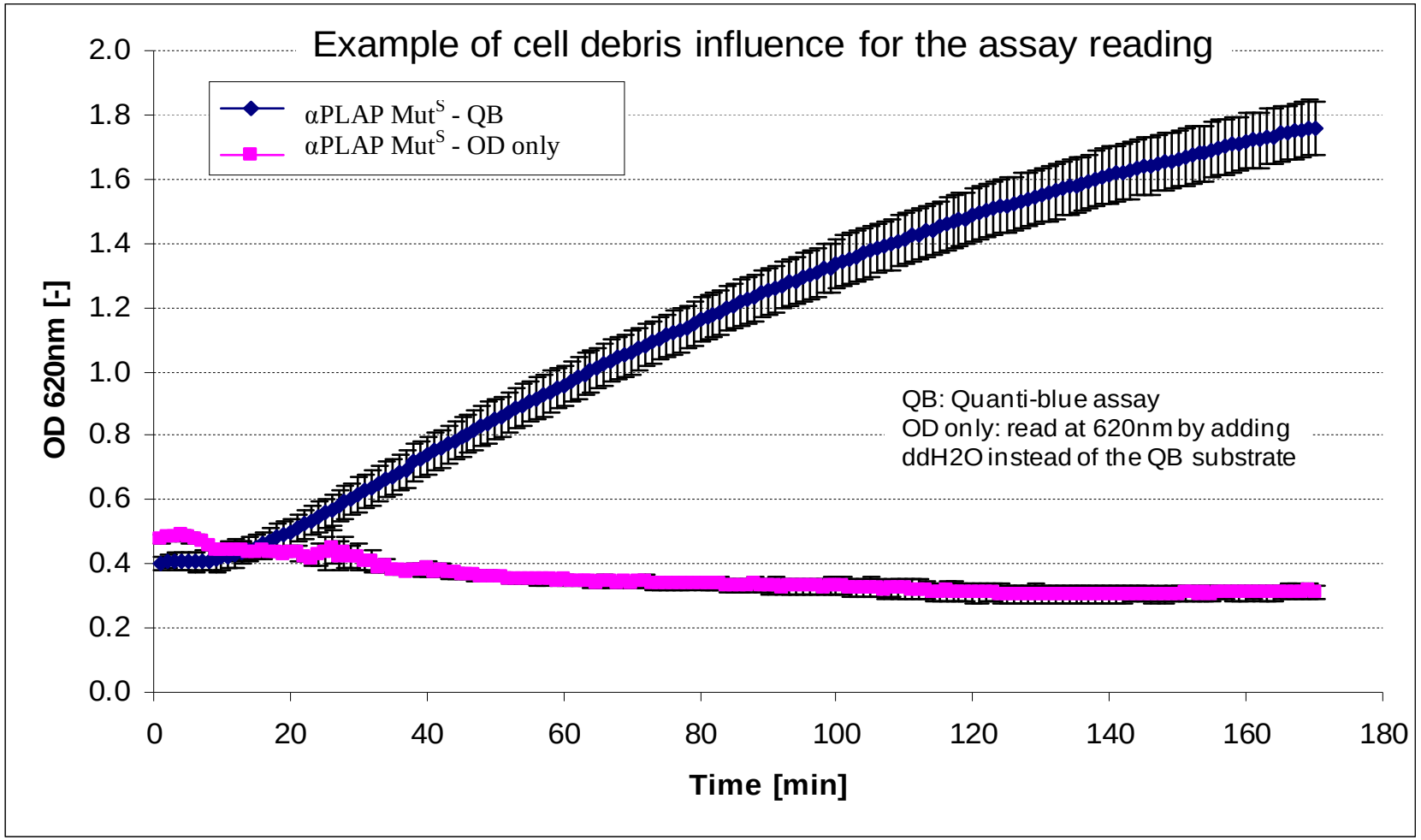


Figure 3.19: An example of debris influence for a Quanti-Blue™ reading
PLAP Quanti-blue™ colorimetric assay for a shake flask sample generated from the culture of *Pichia pastoris* GS115 Mut^S αPLAP. Readings were taken in continuous in a 96-wells plate reader. For biological samples, two readings were required as the colour of the media or the cell debris may influence data. Therefore, a second well was used as a blank, which stabilised within the first hour of reaction, to correct the colorimetric reading.

3.4. Conclusions

Since the aim was to be able to monitor methanol levels below 3.0% (v/v), this assay was unsuitable for the detection of low concentration of methanol in fermenters or shake flasks. In other words, this method was impractical for methanol detection during fermentation as with short reaction times it was not sensitive enough to detect little methanol fluctuactions in biological sample. However, this method could still be used to measure higher methanol concentrations for other applications, to measure methanol concentrations qualitatively to determine presence or absence of methanol (i.e. wine industry), or to analyse residual methanol in fermentation samples with longer reaction times where gas chromatography, for instance, is not available or applicable. Possibly, further studies could be done to try to optimise further this assay and improve its sensitivity by usisng different reaction buffers or reaction catalysers.

As it appeared from the results in this chapter the chemiluminescent assay was of more difficult optimisation because of poor reproducibility. On the other hand, the colorimetric assay had better reproducibility. However, it required longer reaction times, which were then confirmed not to have an influence on protein concentration measurement. Although sensitivity and resolution were greater for the chemiluminescent assay (Yang *et al.*, 1997), the colorimetric one offered a more straightforward optimisation as well as better protein concentration estimation (the chemiluminescent assay tended to overestimate PLAP concentrations). As a result, the latter was chosen as the principal PLAP assay for this project. Since preliminary data on PLAP secretion indicated very small or near zero secretion, and revealed higher protein concentrations

within the cells, all fermentation and shake flasks samples after assay optimisation were performed as outlined in material and method. Both secreted and non-secreted PLAP concentrations were measured and summed. Therefore, all the results related to PLAP yield and concentrations in the next sections were expressed as total PLAP concentration.

4. Construction and initial characterisation of Experimental *Pichia pastoris* strains

The first objective of this work was to create new *Pichia pastoris* strains capable of producing a recombinant protein (placental alkaline phosphatase – PLAP) under the control of the strong AOX1 promoter. Different molecular biology techniques were used to create new strains. In addition different methanol utilisation (Mut) phenotypes were also created (Mut^+ and Mut^S). Construct were then tested and compared for correct gene sequence, insertion, and for accurate phenotype. Shake flasks preliminary studies were done to assess biomass accumulation in conventional (0.5%) and increased (up to 2%) methanol concentrations.

4.1. Construction of P. pastoris cell lines expressing recombinant Placental Alkaline Phosphatase (PLAP)

4.1.1. Vector construction

4.1.1.1. Transformation vector components

pRIS

The pRIS plasmid was build to isolate the hPLAP, *Eco*RI flanked ORF, from previous plasmid backbone (pSEAP2-control). The rationale for the construction of this plasmid was to have the hPLAP gene between two *Eco*RI sites, necessary for insertion into the parent plasmid pAO815 (figure 1.6) obtained commercially from Invitrogen Corp. (San Diego, CA, USA). The human secretion signal (5'-CTGCTGCTGCTGCTGCTGCTGGGCCTGAGGCTACAGCTCTCCC TGGGCATC –3') was excised from the pSEAP2-control vector (Clontech Laboratories, Inc., Saint-Germain-en-Laye, France). This plasmid has been previously constructed at UCL, Biochemical Engineering Department.

The PLAP gene was excised from the pRIS vector (figure 4.1) by digestion with the enzyme *Eco*RI as outlined in material and methods for single enzyme digestion (mini). After digestion, samples showing a 1636 bp band (PLAP gene) on electrophoresis gel were considered positive (figure 4.2). DNA in these bands has been extracted and purified from gel and used for ligation with the linearised (*Eco*RI cut) pAO815.

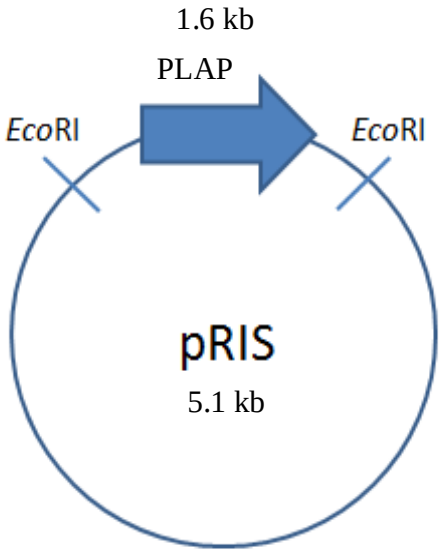


Figure 4.1: pRIS draw
pRIS vector containing the PLAP gene flanked between two EcoRI restriction sites.

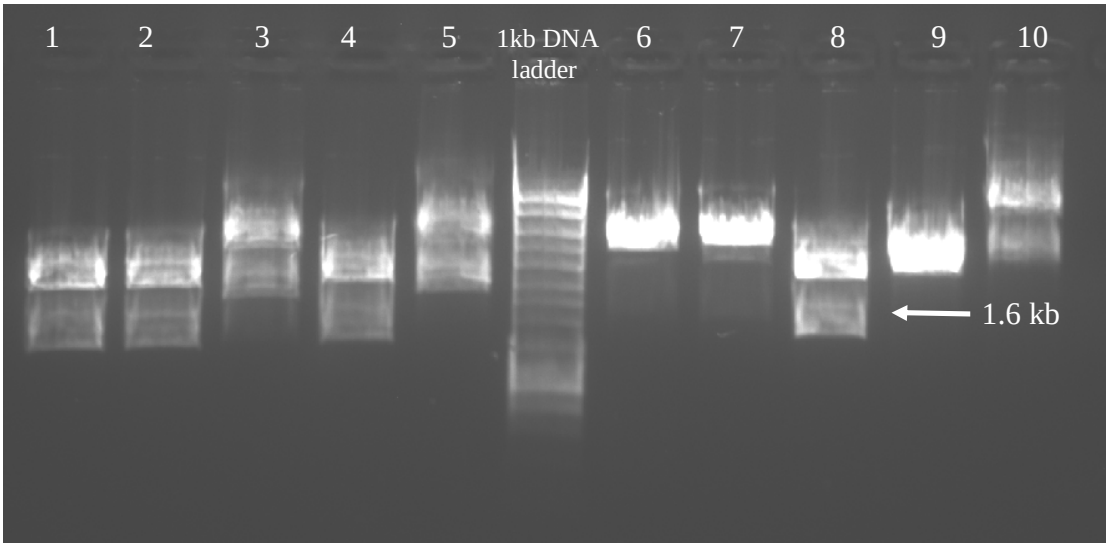


Figure 4.2: Electrophoresis of EcoRI-digested pRIS
Electrophoresis gel showing pRIS patterns after digestion with the enzyme *Eco*RI. Samples 1, 2, 4, and 8 were positive (1.6kb band). 1kb DNA ladder band sizes (from top to bottom): 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1.5 kb, 1kb, 0.5kb.

pAO815

The pAO815 plasmid was digested and linearised with *Eco*RI (figure 4.3) as outlined in material and methods.

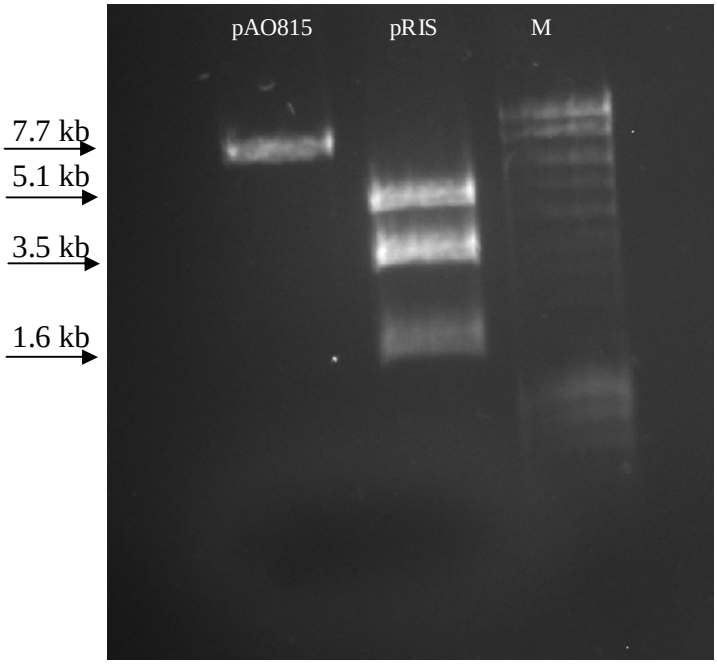


Figure 4.3: Electrophoresis of EcoRI-digested pRIS and pAO815
This electrophoresis gel showed that pAO815 has been completely linearised by *Eco*RI. The three fragments visible on the pRIS digest are a 1.6 kb fragment (PLAP), the pRIS-PLAP minus the PLAP fragment, and the linearised plasmid (pRIS-PLAP), indicating not complete digestion. However, fragments were taken directly by cutting the gel in order to reduce contamination from other fragments, so another digestion was not needed. M: 1kb DNA ladder band sizes (from top to bottom): 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1kb, 0.5kb.

4.1.1.2. Vector assembly and cloning strategy

Cloning Strategy

The strategy used to construct the pAO36 vector is outlined in figure 4.4.

pAO36 fragment ligation

Once the pAO815 linearised plasmid and the hPLAP fragment were isolated, they were ligated together to generate the pAO36 plasmid, needed to transform *Pichia pastoris* GS115 wild-type and, therefore generate the new clones. This ligation was performed at room temperature (30 minutes) and left in fridge overnight. Samples composition is shown in table 4.1.

Table 4.1: pAO36 ligation calculations
Composition of samples for the ligation of the PLAP fragment and the linearised pAO815.

Sample	3:1	1:1	C1	C2
Quick Ligase (T4)	1	1	1	0
2x Buffer	10	10	10	10
pAO815-*Eco*RI cut	2	2	2	2
pRIS-*Eco*RI cut	7	2.5	0	0
dH_2O	0	4.5	7	8
	20 µl	**20 µl**	**20 µl**	**20 µl**

Note: 3:1 and 1:1 indicate the ratio used for transformation (pRIS-*Eco*RI cut:pAO815-*Eco*RI cut). C1 and C2 are the controls.

E. coli transformation

E. coli cells were transformed with pAO36 as outlined in material and methods. The 3:1 ratio mixture performed better in terms of transformation efficiency. In addition to the 4 combinations described above (table 4.1), two positive and negative transformation controls (i.e. *E. coli* with no DNA, *E. coli* transformed with pUC18) were added. The obtained results are outlined in table 4.2.

Table 3.2: *E. coli* – pAO36 Transformation outcome

Sample	Plate count
***E.coli* + 3:1**	186
***E.coli* + 1:1**	177
***E.coli* + C1**	59
***E.coli* + C2**	27
***E.coli* + pUC18**	> 200
***E.coli* + ddH_2O**	0

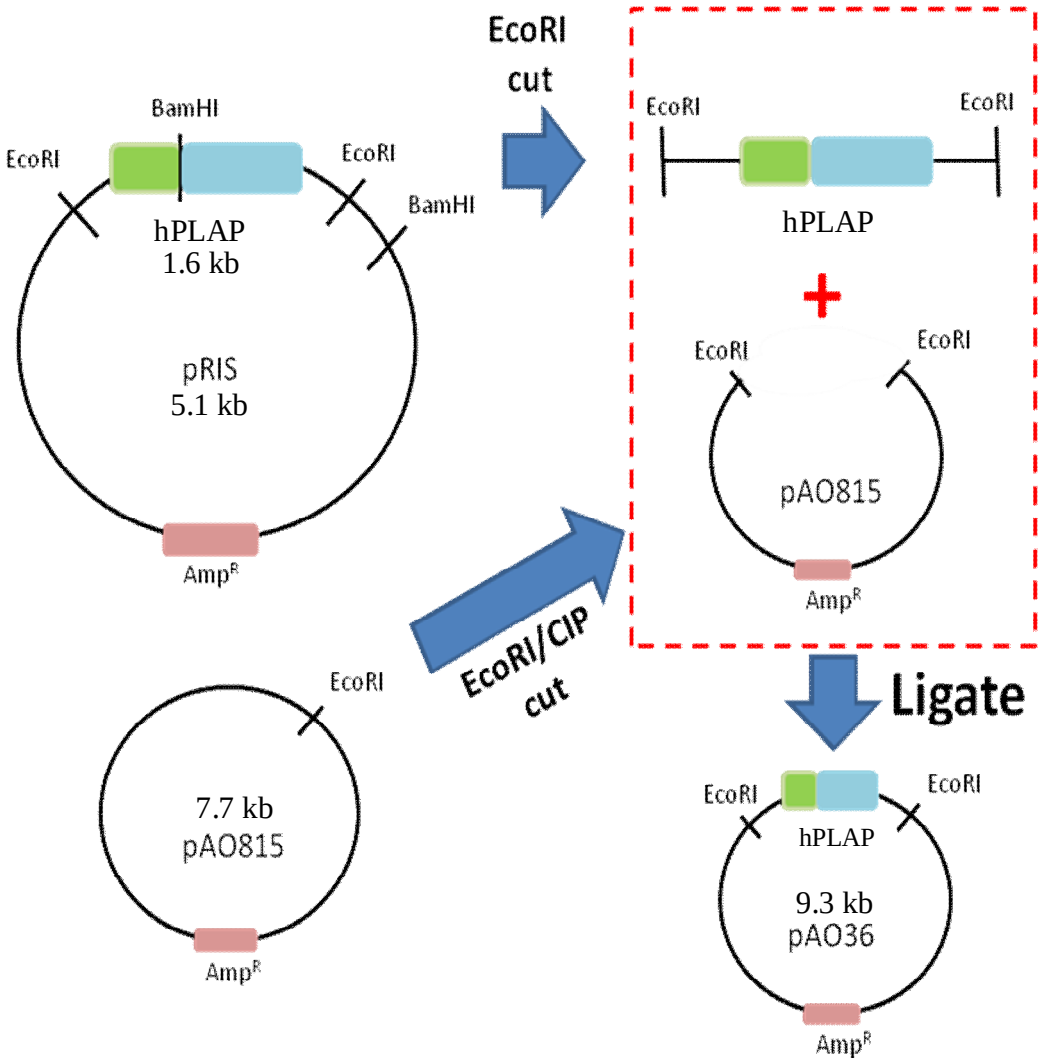


Figure 4.4: Cloning strategy for the construction of pAO36
Strategy used for the construction of a PLAP-carrying vector having a human secretion signal (hPLAP) for the transformation of *Pichia pastoris* GS115 wild-type. The resulting plasmid was originated from the ligation of hPLAP with the parent plasmid pAO815. pAO815 from Invitrogen Corporation is a typical *Pichia pastoris* expression vector. Its main features are the *AOX1* promoter and transcriptional terminator, the *Pichia pastoris* histidinol dehydrogenase gene for *his4* hosts selection, the *E. coli* pBR322 origin of replication and ampicillin resistance, and a unique restriction site (*Eco*RI) for insertion of heterologous proteins. The 3' *AOX1* sequence is aimed to target plasmid integration at the *AOX1* gene locus, helped by a linearization of the plasmid with *Sal*I or *Stu*I (insertion at *HIS4* – generates *His*$^+$ Mut$^+$ in *Pichia pastoris* GS115) or with *Bgl*II (gene replacement at *AOX1* – generates *His*$^+$ Mut^S in *Pichia pastoris* GS115), which stimulate single-crossover type integration events (Cregg *et al.*, 1993).

4.1.1.3. Vector characterisation and confirmation

E. coli colonies were tested for the PLAP fragment presence by a *Eco*RI digestion of the extracted DNA. Among all the tested colonies (40), 12 were found positive (figure 4.5, not all samples showed).

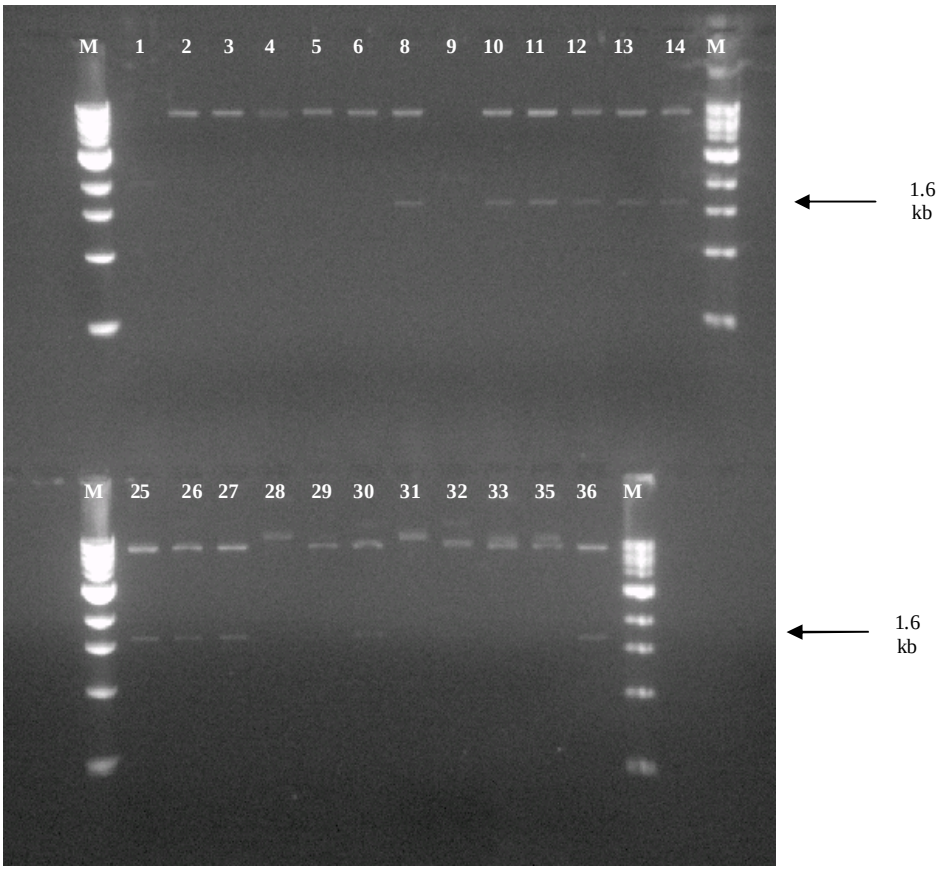


Figure 4.5: Electrophoresis of EcoRI-digested pAO36
Electrophoresis gels generated from the nucleic acid extraction of the pAO36 vector from transformed *E. coli* cells and digested with *Eco*RI. 1-36: Samples; M: 1 kb DNA ladder, band sizes (from top to bottom): 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1.5 kb, 1kb, 0.5kb.

Because of the *Eco*RI digestion on both sides of the sequence, the possibility (50%) of having the gene inserted in the wrong orientation make it necessary a control digestion to determine the hPLAP orientation. After digestion with *Pst*I two patterns should be visible on electrophoresis gels: Fragments of 7.4 kb and 1.9 kb indicate the right orientation; Fragments of 6.0 kb and 3.3 kb indicate the wrong orientation (figures 4.6-4.7). Among the tested samples 3 were found to have the right orientation. Clone 36 was then used.

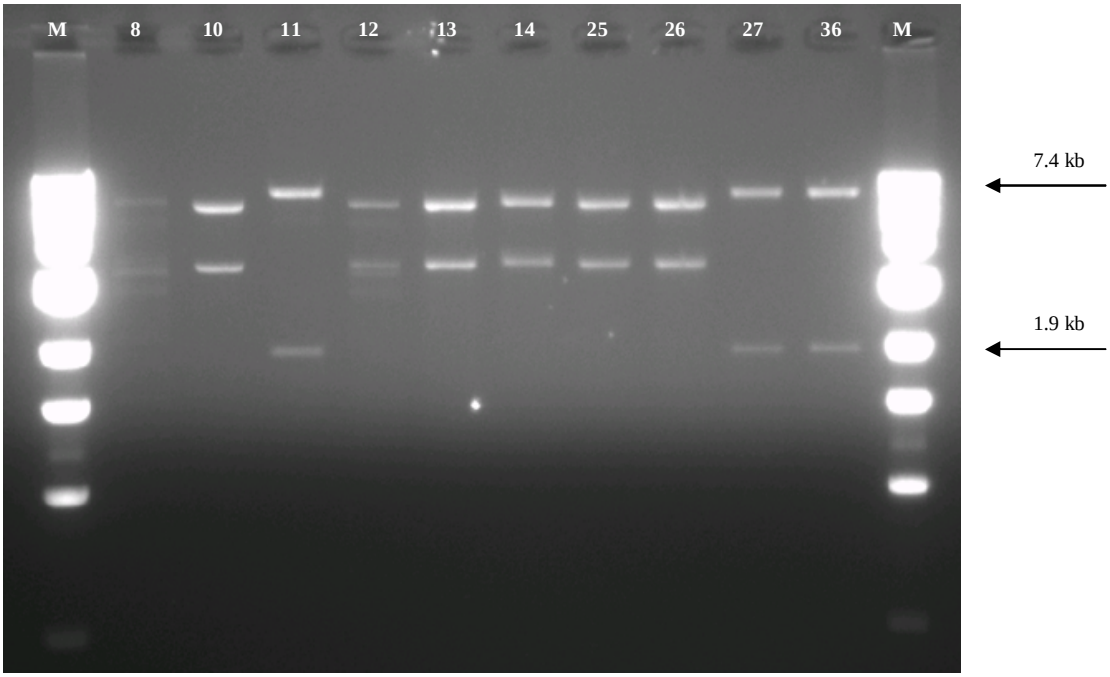


Figure 4.6: Electrophoresis of the PstI orientation digestion of pAO36
Electrophoresis gels generated from the orientation digestion of the pAO36 vector with *Pst*I. 8, 10-14, 25-27, and 36: Samples; M: 1 kb DNA ladder, band sizes (from top to bottom): 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1.5 kb, 1kb, 0.5kb.

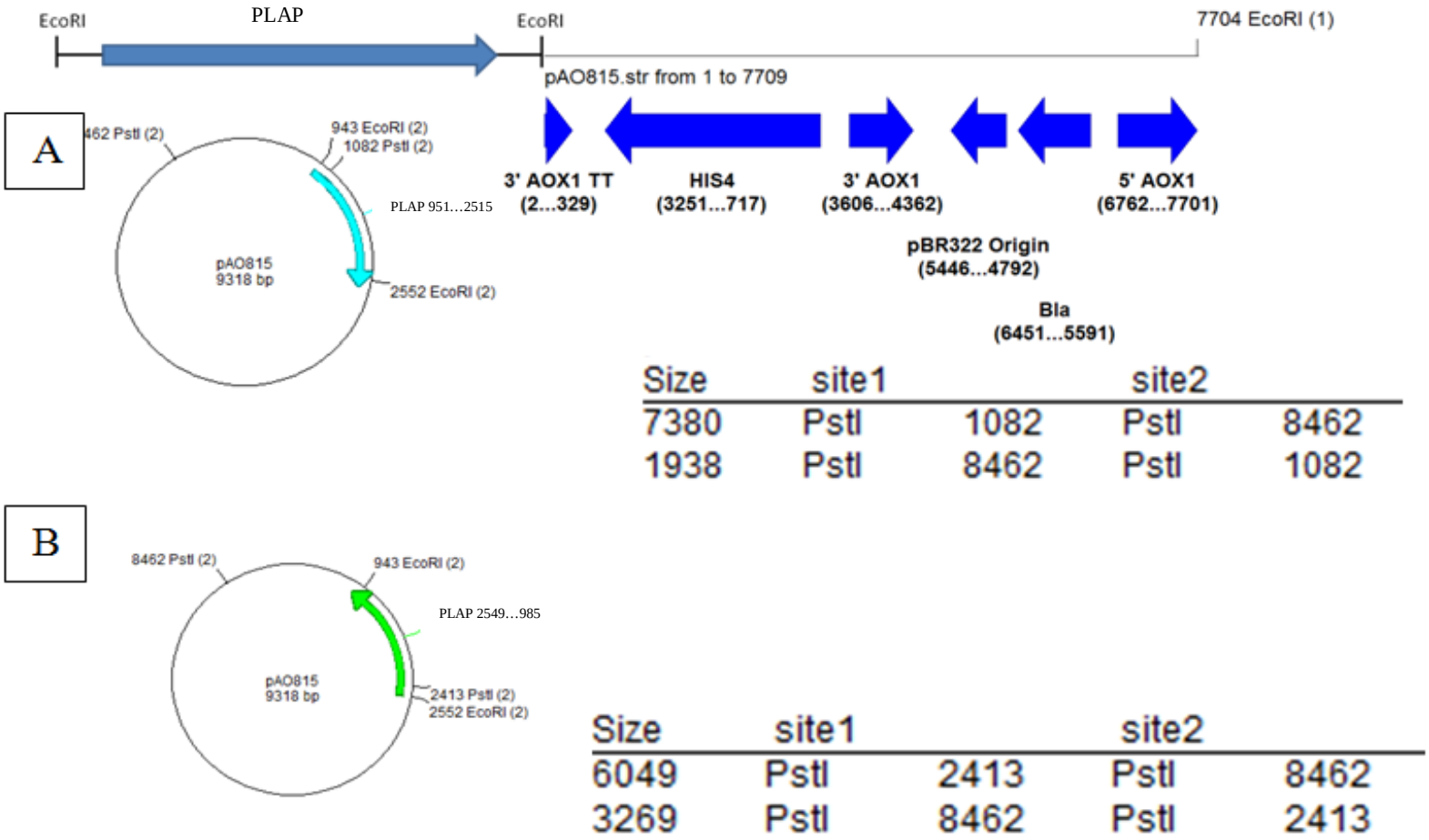


Figure 4.7: Possible pAO36 orientation digestion outcomes
This figure shows the two possible orientations generated from the ligation of the PLAP fragment with the linearised pAO815 vector. After digestion with *Pst*I two patterns should be visible on electrophoresis gels: 1. Fragments of 7.4 kb and 1.9 kb indicate the right orientation (A); 2. Fragments of 6.0 kb and 3.3 kb indicate the wrong orientation (B).

To confirm correct gene orientation and sequence, five oligonucleotides were designed (figures 4.8) for sequencing experiments (hPLAP-f250:5'-ATACCCCTGGCCATGGACC GC; hPLAP-f750: 5'-CAGGAATGG CTGGCGAAGCGC; hPLAP-f1200: 5'-GGCTATGTGCTCAAGGACGGC; hPLAP-r500: 5'-GACTTCCCTGCTTT CTTGGCC; hPLAP-r1000: 5'-CAGTC AGTGCCCGGTAAGCCC). The DNA sequencing (figure 4.9) has been performed for us by the Wolfson Institute, University College of London, London, UK.

ATGCTGCTGCTGCTGCTGCTGCTGGGCCTGAGGCTACAGCTCTCCCTGGGCATCATCCCAGTTG
AGGAGGAGAACCCGGACTTCTGGAACCGCGAGGCAGCCGAGGCCCTGGGTGCCGCCAAGAA
GCTGCAGCCTGCACAGACAGCCGCCAAGAACCTCATCATCTTCCTGGGCGATGGGATGGGGG
TGTCTACGGTGACAGCTGCCAGGATCCTAAAAGGGCAGAAGAAGGACAAACTGGGGCCTGAG
ATACCCCTGGCCATGGACCGCTTCCCATATGTGGCTCTGTCCAAGACATACAATGTAGACAAA
CATGTGCCAGACAGTGGAGCCACAGCCACGGCCTACCTGTGCGGGGTCAAGGGCAACTTCCA
GACCATTGGCTTGAGTGCAGCCGCCCGCTTTAACCAGTGCAACACGACACGCGGCAACGAGG
TCATCTCCGTGATGAATCGGGCCAAGAAAGCAGGGAAGTCAGTGGGAGTGGTAACCACCACA
CGAGTGCAGCACGCCTCGCCAGCCGGCACCTACGCCCACACGGTGAACCGCAACTGGTACTC
GGACGCCGACGTGCCTGCCTCGGCCCGCCAGGAGGGGTGCCAGGACATCGCTACGCAGCTCA
TCTCCAACATGGACATTGACGTGATCCTAGGTGGAGGCCGAAAGTACATGTTTCGCATGGGAA
CCCCAGACCCTGAGTACCCAGATGACTACAGCCAAGGTGGGACCAGGCTGGACGGGAAGAAT
CTGGTGCAGGAATGGCTGGCGAAGCGCCAGGGTGCCCGGTATGTGTGGAACCGCACTGAGCT
CATGCAGGCTTCCCTGGACCCGTCTGTGACCCATCTCATGGGTCTCTTTGAGCCTGGAGACAT
GAAATACGAGATCCACCGAGACTCCACACTGGACCCCTCCCTGATGGAGATGACAGAGGCTG
CCCTGCGCCTGCTGAGCAGGAACCCCCGCGGCTTCTTCCTCTTCGTGGAGGGTGGTCGCATCG
ACCATGGTCATCATGAAAGCAGGGCTTACCGGGCACTGACTGAGACGATCATGTTCGACGAC
GCCATTGAGAGGGCGGGCCAGCTCACCAGCGAGGAGGACACGCTGAGCCTCGTCACTGCCGA
CCACTCCCACGTCTTCTCCTTCGGAGGCTACCCCCTGCGAGGGAGCTCCATCTTCGGGCTGGCC
CCTGGCAAGGCCCGGGACAGGAAGGCCTACACGGTCCTCCTATACGGAAACGGTCCAGGCTA
TGTGCTCAAGGACGGCGCCCGGCCGGATGTTACCGAGAGCGAGAGCGGGAGCCCCGAGTATC
GGCAGCAGTCAGCAGTGCCCCTGGACGAAGAGACCCACGCAGGCGAGGACGTGGCGGTGTTC
GCGCGCGGCCCGCAGGCGCACCTGGTTCACGGCGTGCAGGAGCAGACCTTCATAGCGCACGT
CATGGCCTTCGCCGCCTGCCTGGAGCCCTACACCGCCTGCGACCTGGCGCCCCCCGCCGGCAC
CACCGACGCCGCGCACCCGGGTTACTCTAGAGTCGGGGCGGCCGGCCGCTTCGAGCAGACAT
GA

250bp 750bp 1200bp
> > >
_____ 1560bp PLAP ORF
< <
500bp 1000bp

Primers

Forward
Position 250: ATACCCCTGGCCATGGACCGC
Position 750: CAGGAATGGCTGGCGAAGCGC
Position 1200: GGCTATGTGCTCAAGGACGGC

Reverse
Position 500: GACTTCCCTGCTTTCTTGGCC
Position 1000: CAGTCAGTGCCCGGTAAGCCC

Figure 4.8: PLAP gene PCR primers design
This figure shows the 1560bp PLAP ORF sequence and forward (red) and reverse (green) primers sequence and position.

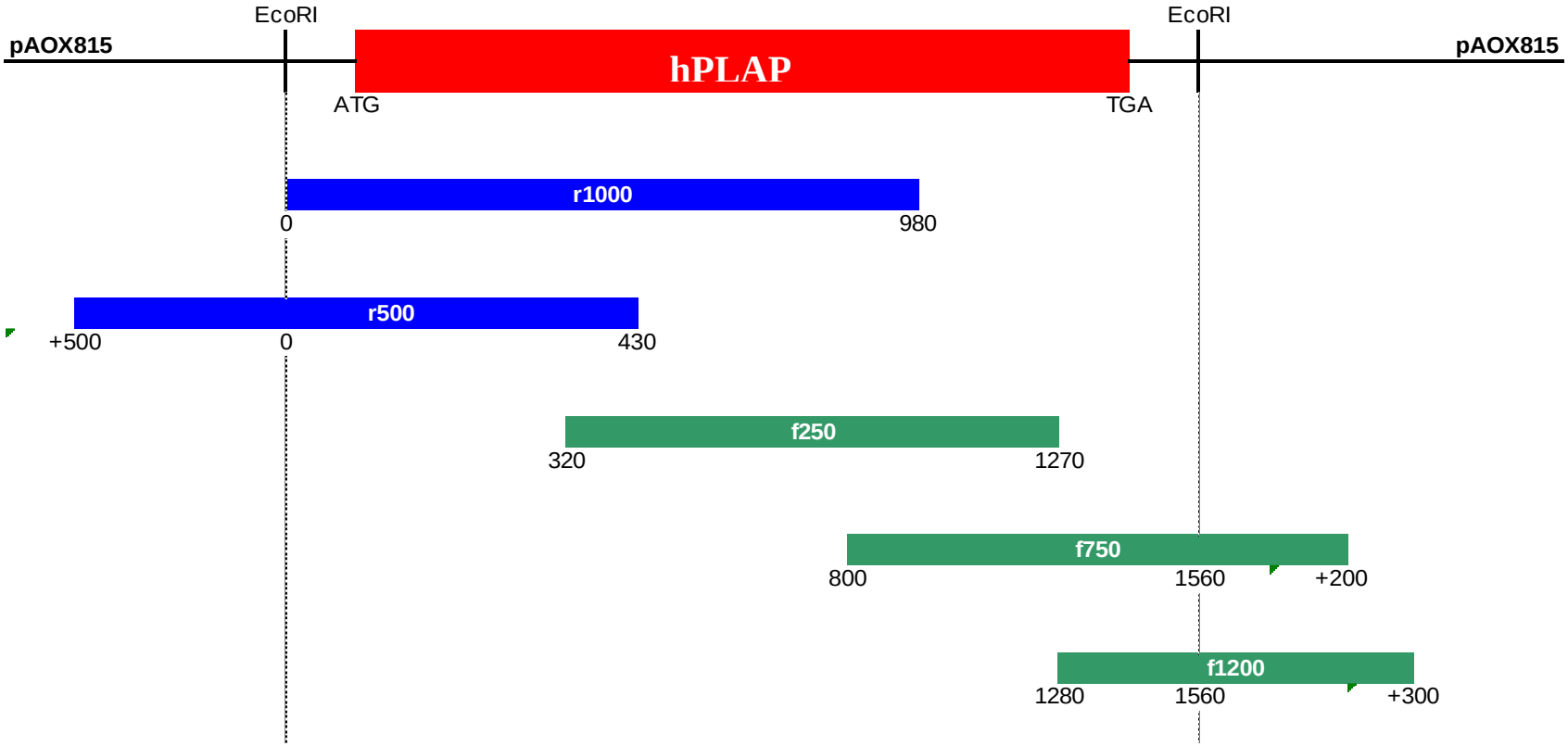


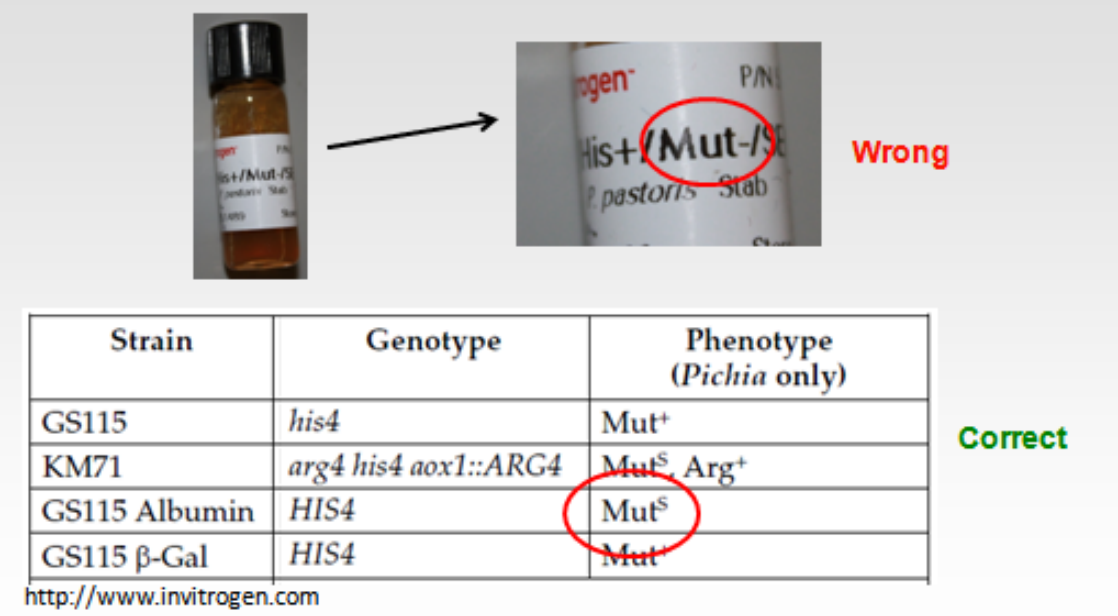
Figure 4.9: PLAP sequencing alignment outcome
This figure shows the alignment of the sequences obtained from the 5 primers described in this section with the pAO815 parent plasmid having the hPLAP gene inserted (pAO36) in the right orientation and correct sequence.

4.1.2. *P. pastoris* transformation

4.1.2.1. Transforming *P. Pastoris* GS115 Wild-Type (WT) with pAO36

Construction of *Pichia pastoris* GS115 HIS4 Mut$^+$ hPLAP by electroporation

The constructed pAO36 was linearised with the enzyme *Sal*I (figure 4.11). DNA was purified according to material and methods to remove all traces of PEG. Electrocompetent *Pichia pastoris* GS115 wild-type cells were then transformed as described in the material and methods section. After incubation, integration was tested by PCR, whereas the Mut phenotype was checked by plating colonies on YPD, MD, and MM plates divided in grids of 16 squares. Here a mislabelling has been found for the Invitrogen's cells used as growth controls. In fact, a *Pichia pastoris* GS115 (Human Serum Albumin) strain supposed to act a Mut$^-$ (no growth on methanol) was growing on methanol. After repeating the experiments and doing some more research on this strain to exclude contamination, otherwise strains could not be released for further use, was found that this strain was in fact Mut^S and not Mut$^-$ as stated on the label (figure 4.10).



Strain	Genotype	Phenotype (*Pichia* only)
GS115	*his4*	Mut^{+}
KM71	*arg4 his4 aox1::ARG4*	~~Mut^{S}~~, Arg^{+}
GS115 Albumin	*HIS4*	Mut^{S}
GS115 β-Gal	*HIS4*	~~Mut^{+}~~

Figure 4.10: *Pichia pastoris* GS115 HSA mis-labelling
Mislabelling of *Pichia pastoris* GS115 Albumin (HSA) obtained commercially from Invitrogen's corporation.

Construction of *Pichia pastoris* GS115 HIS4 Mut^{S} hPLAP by electroporation

DNA (pAO36) was linearised with the enzyme *Bgl*II (figure 4.11). The same phenotype procedure as that used for *Pichia pastoris* GS115 His^{+} Mut^{+} hPLAP strain was used to confirm *Pichia pastoris* GS115 His^{+} Mut^{S} hPLAP.

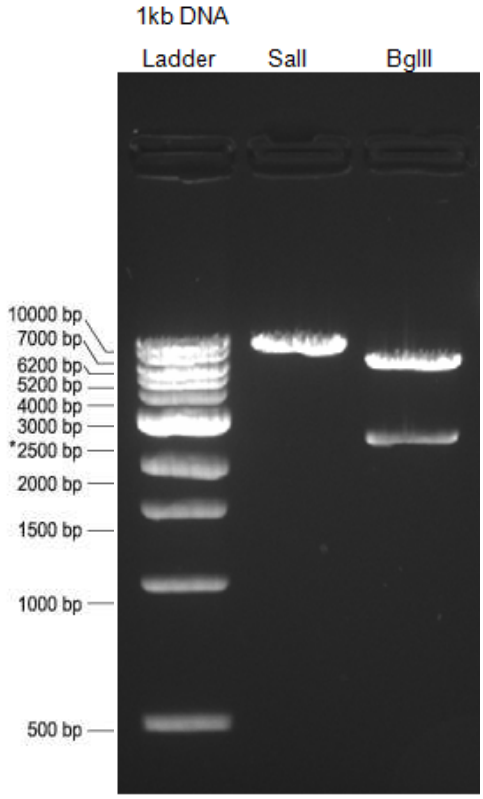


Figure 4.11: Electrophoresis of the pAO36 linearisation with SalI and BglII
This electrophoresis gel showed complete digestion of the pAO36 plasmid with both *Sal*I and *Bgl*II enzymes giving a 9.3 kb band and 2.4 kb and 6.9 kb bands, respectively.

4.1.2.2. Strains' characterisation and confirmation

Confirmation of hPLAP integration into the *Pichia pastoris'* genome by PCR

Chromosomal integration of the hPLAP gene into the *Pichia pastoris* GS115 genome was confirmed by PCR. Two primers (hPLAP-f250 and hPLAP-r1000 – figures 4.8 and 4.9) should amplify a 0.66 kb fragment between positions 320 and 980 in the hPLAP sequence. One colony of each *Pichia pastoris* GS115 wild-type, hPLAP Mut^{+}, and hPLAP Mut^{S} from YPD plates was added to 100 µl of ddH_2O. Samples were heated

for 10 minutes at 95˚C in order to disrupt cells. 1 µl of the generated suspension was used for PCR, which was performed according to the protocol outlined in the material and methods section. In addition of the three samples described above, purified pAO36 and ddH_2O were used as positive and negative controls, respectively. Samples were then visualised by electrophoresis (figure 4.12) as previously described. In order to exclude DNA carryover from transformation to PCR, all the dilutions steps cells went through were calculated and less than 2.3E-14 fg/L of DNA was found to remain in the samples from transformation to PCR, and therefore false positive results were to be excluded.

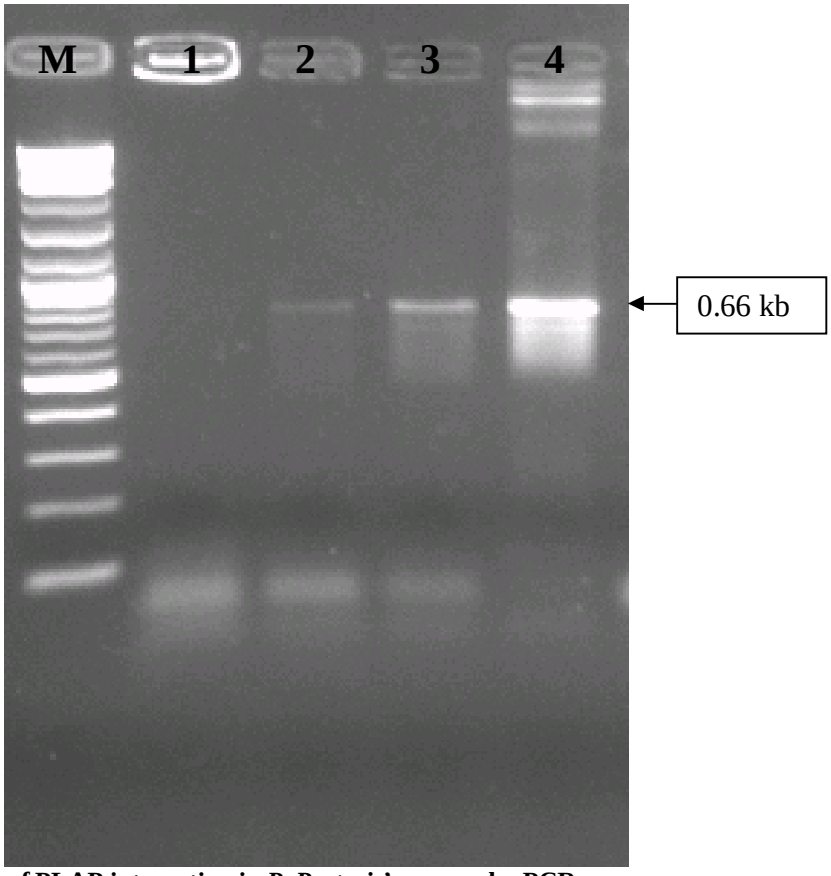


Figure 4.12: Confirmation of PLAP integration in *P. Pastoris'* genome by PCR
This electrophoresis gel showed the 0.66 kb fragments amplified by PCR to confirm plasmid integration (pAO36) into *Pichia pastoris* GS115 wild-type. M: 100 bp DNA Ladder, 1: ddH_2O, 2: *Pichia pastoris* GS115 HIS4 Mut^+ hPLAP, 3: *Pichia pastoris* GS115 HIS4 Mut^S hPLAP, 4: pAO36.

Confirmation of the Mut phenotype for Mut^+ and Mut^S strains

In order to screen Mut^+ and Mut^S transformants strains were characterised against control strains in MM media dishes divided into 16 squares, as well as MD and YPD plates divided in the same way (tables 4.3, 4.4, and 4.5). The results, quantified in Figure 4.13, showed that Mut^+ cells grew faster and were visible on plate after one day incubation at 30˚C. However, Mut^S cells appeared on plate after 2 days incubations at 30˚C, indicating slower methanol utilisation and therefore confirming the phenotype (Figure 4.13).

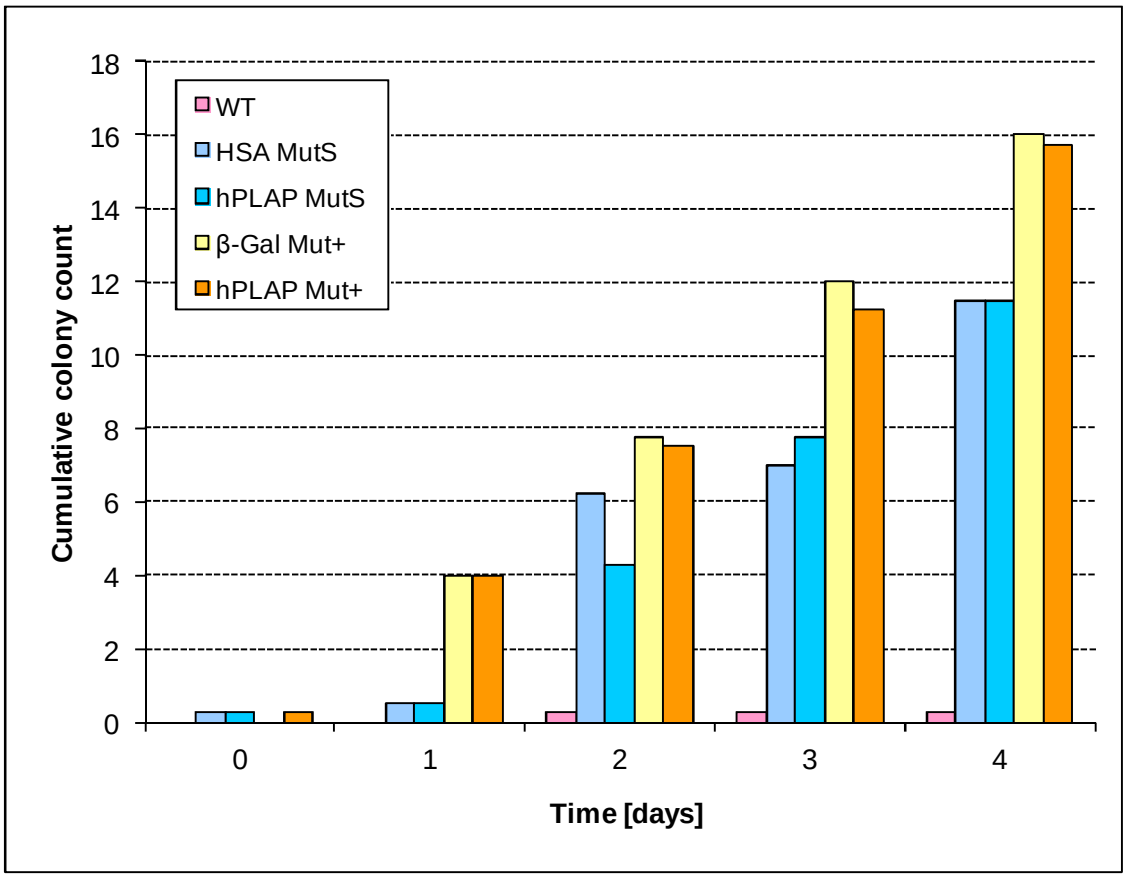


Figure 4.13: *P. Pastoris* growth comparison in MM-agar media
Cumulative growth of different strains of *Pichia pastoris* GS115 in MM media. This graph showed that Mut^+ strains grow faster than Mut^S strains in methanol-containing media. WT has his4 phenotype, therefore it was, as expected, unable to grow on MM media. All other strains were HIS4, thus they can grow on MM media without histidine supplement. This graph has been generated by scoring (by the naked eye) the colonies grown on plates, each having a value related to the colony size.

Table 4.3: *Pichia pastoris* GS115 YPD grid growth control
All *Pichia pastoris* GS115 strains must grow in YPD media

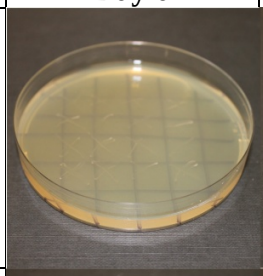
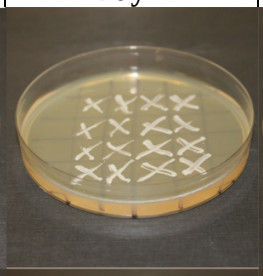
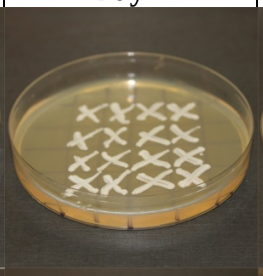
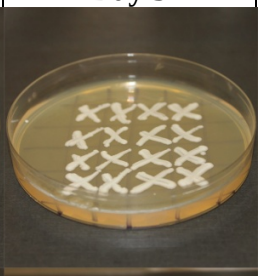
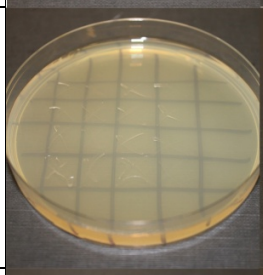
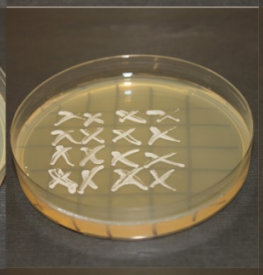
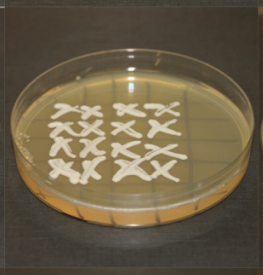
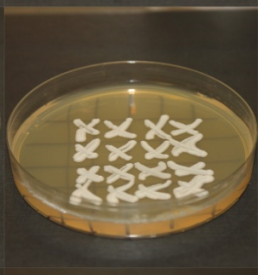
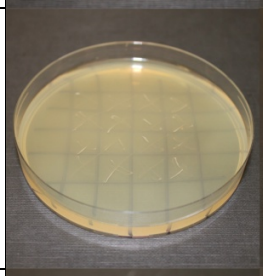
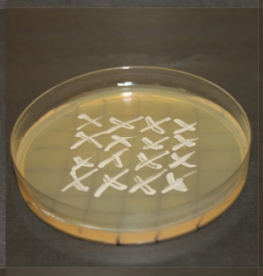
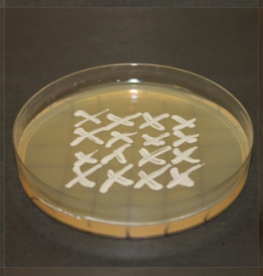
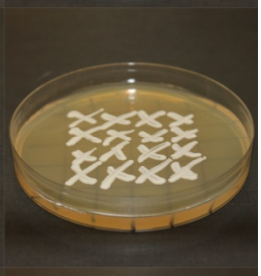
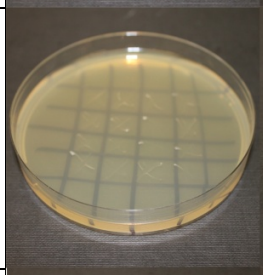
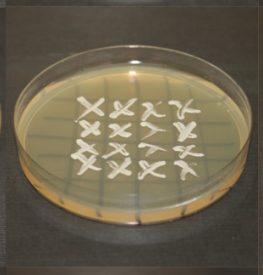
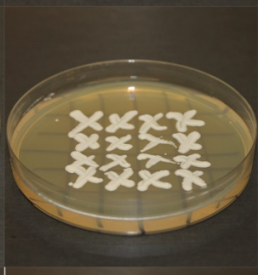
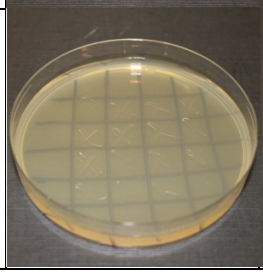
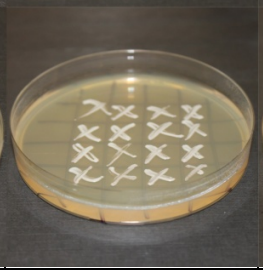
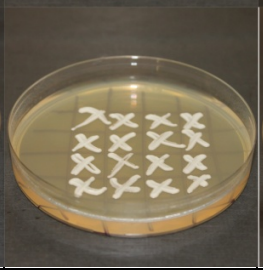
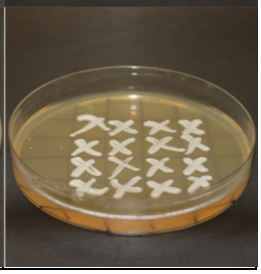
Strain	Day 0	Day 1	Day 2	Day 3
P. pastoris GS115 Wild-type (his4)				
P. pastoris GS115 HSA HIS4 Mut^{S}				
P. pastoris GS115 β-Gal HIS4 Mut^{+}				
P. pastoris GS115 hPLAP HIS4 Mut^{+}				
P. pastoris GS115 hPLAP HIS4 Mut^{S}				

Table 4.4: *Pichia pastoris* GS115 HIS4 phenotype grid control
All HIS4 *Pichia pastoris* GS115 strains must grow in MD media, his4 strains (WT) require histidine supplemented to the media.

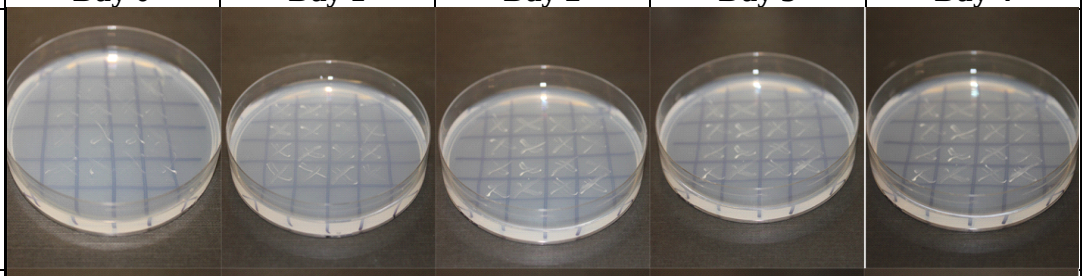
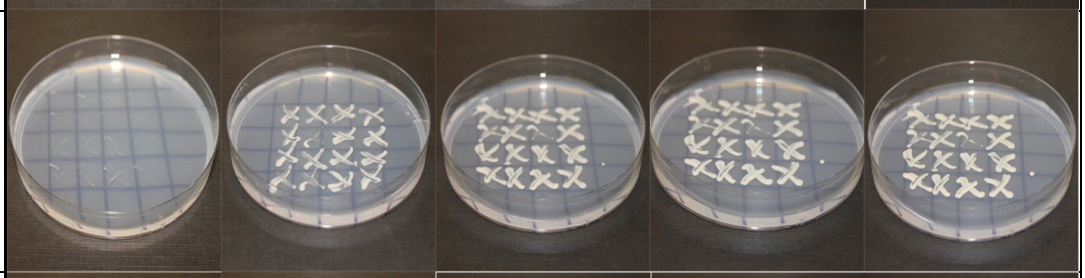
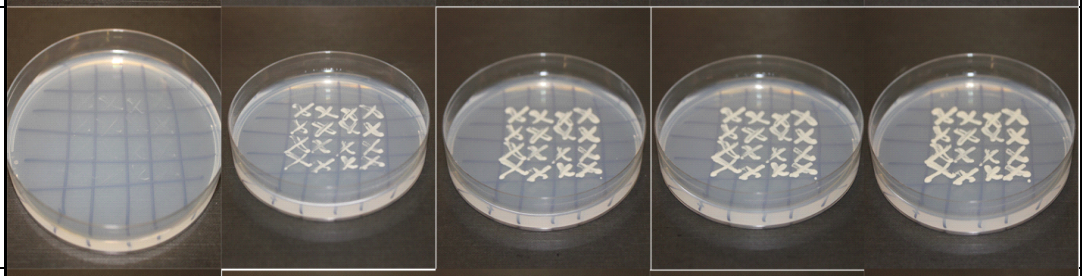
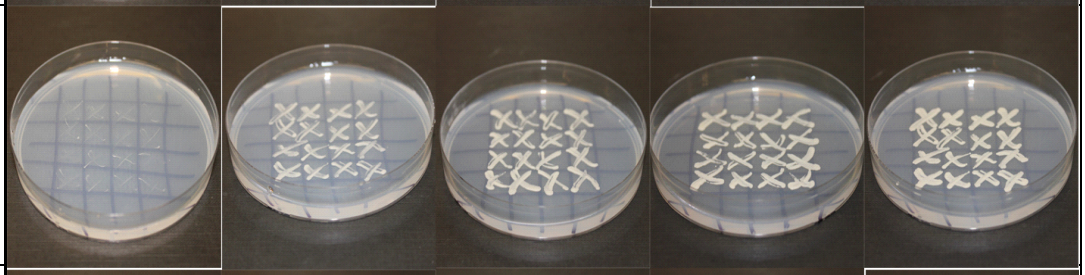
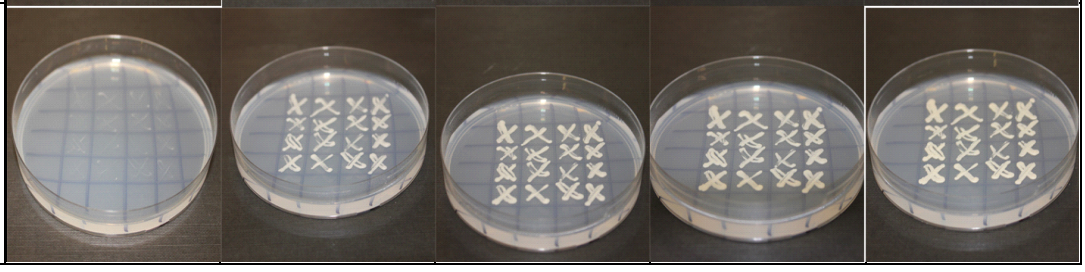
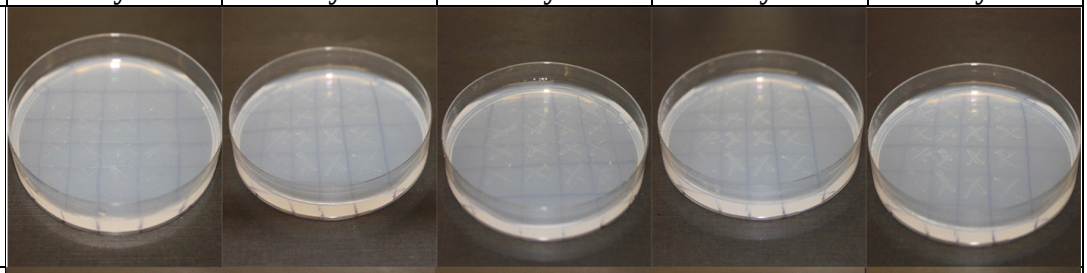
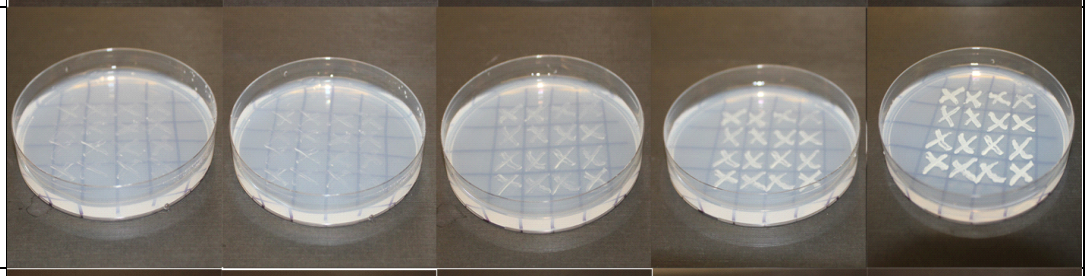
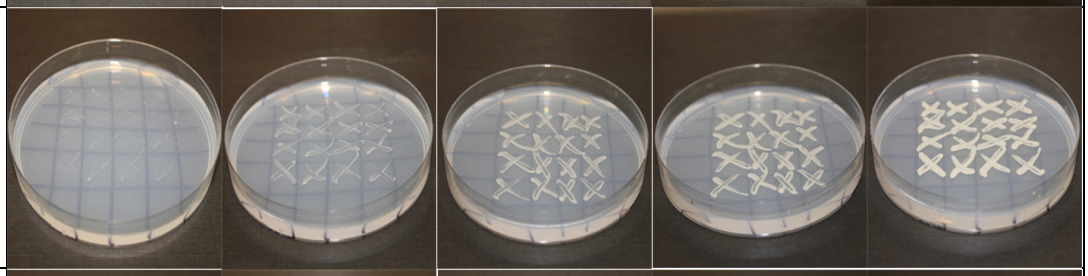
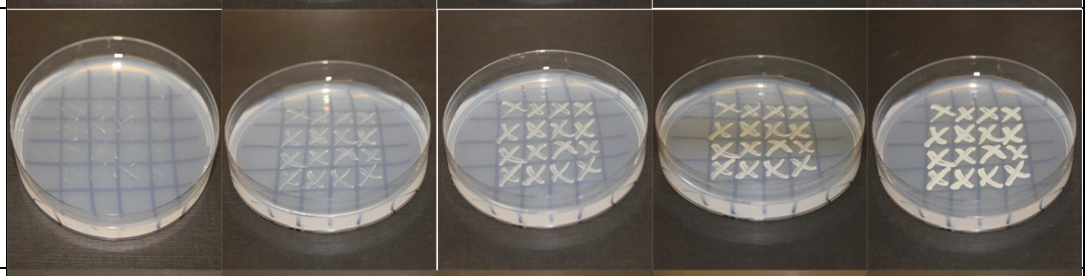
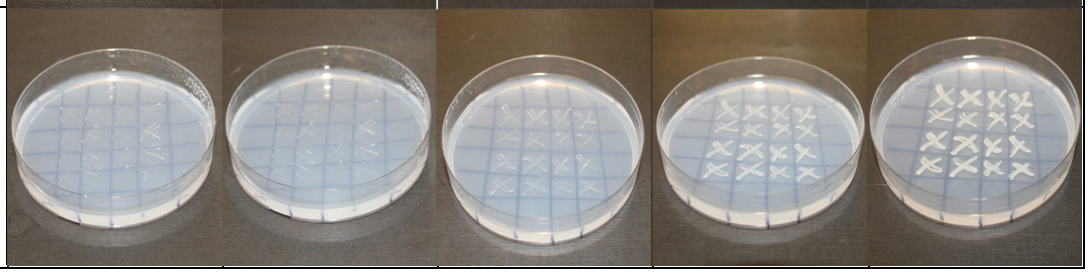
Strain	Day 0	Day 1	Day 2	Day 3	Day 4
P. pastoris GS115 Wild-type (his4)					
P. pastoris GS115 HSA HIS4 Mut^S					
P. pastoris GS115 β-Gal HIS4 Mut^+					
P. pastoris GS115 hPLAP HIS4 Mut^+					
P. pastoris GS115 hPLAP HIS4 Mut^S					

Table 4.5: *Pichia pastoris* GS115 Mut phenotype grid control
All HIS4 *Pichia pastoris* GS115 strains must grow in MM media, his4 strains (WT) require histidine supplemented to the media. MM media allows to discriminate between Mut^+ and Mut^S strains as the former should grow faster in media containing methanol as a sole source of carbon.

Strain	Day 0	Day 1	Day 2	Day 3	Day 4
P. pastoris GS115 Wild-type (his4)					
P. pastoris GS115 HSA HIS4 Mut^S					
P. pastoris GS115 β-Gal HIS4 Mut^+					
P. pastoris GS115 hPLAP HIS4 Mut^+					
P. pastoris GS115 hPLAP HIS4 Mut^S					

4.2. Construction of a P. pastoris strain expressing αPLAP

Two *P. pastoris* GS115 HIS4 αPLAP strains (Mut^+ and Mut^S) have been previously constructed at UCL, Department of Biochemical Engineering, London, UK. The same procedure used to construct *Pichia pastoris* GS115 HIS4 $Mut^{+/S}$ hPLAP was used. An outline of the vector (pPSS) used for transformation can be found in figure 4.14.

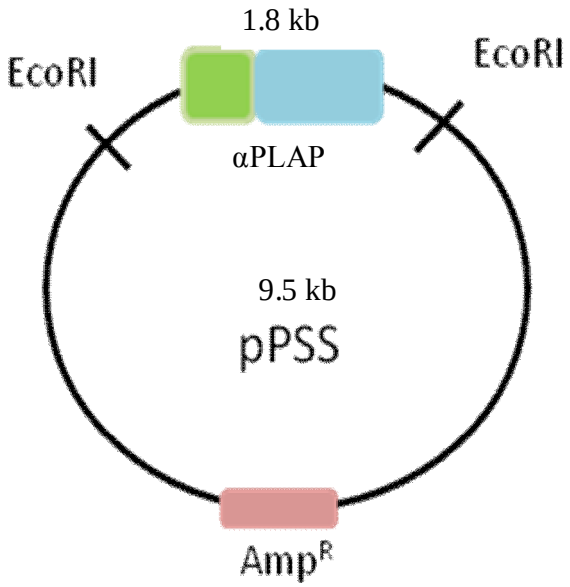


Figure 4.14: pPSS vector map
This plasmid has been previously constructed at UCL, Department of Biochemical Engineering, London, UK. This vector carries an Ampicillin resistance gene and the PLAP gene coupled with a yeast secretion signal (αPLAP) flanked between two EcoRI sites. The 3' *AOX1* sequence is aimed to target plasmid integration at the *AOX1* gene locus, helped by a linearization of the plasmid with *Sal*I or *Stu*I (insertion at *HIS4* – generates *His*$^+$ Mut^+ in *Pichia pastoris* GS115) or with *Bgl*II (gene replacement at *AOX1* – generates *His*$^+$ Mut^S in *Pichia pastoris* GS115), which stimulate single-crossover type integration events (Cregg *et al.*, 1993).

4.3. Strains confirmation and comparison

Results from figure 4.12 have been repeated after two years incubation at -80°C to confirm once more presence of the PLAP gene in the new strains of *Pichia pastoris* GS115 HIS4 $Mut^{+/S}$ hPLAP. In addition, *Pichia pastoris* GS115 HIS4 $Mut^{+/S}$ αPLAP strains were also tested. Results showed PLAP presence in all the tested strains (Figure 4.15). Growth curves (Figure 4.16) showed different growth profiles in glycerol in shake flasks. However, this differences were due to different cell concentrations in the inoculum vial from the cell banks. Growth on methanol up to 1% methanol concentration (first 2 days) was similar for all strains except for the wild-type as histidine was not supplemented in the media with methanol. In liquid shake flask cultures, differences between Mut^S and Mut^+ strains were not visible in terms of growth in methanol (below 1% v/v). After the second day in methanol, and at methanol concentrations over 1.5%, Mut^+ strains performed better (as expected) than Mut^S strains. However, over 2% all strains had difficulties growing on methanol. Cell age may also play a role in OD reduction and cell death, but Figure 4.17 showed continuous growth after 4 days in methanol at a constant methanol concentration of 0.5% (v/v). This suggested that methanol concentrations over 2% have a negative effect on cell growth.

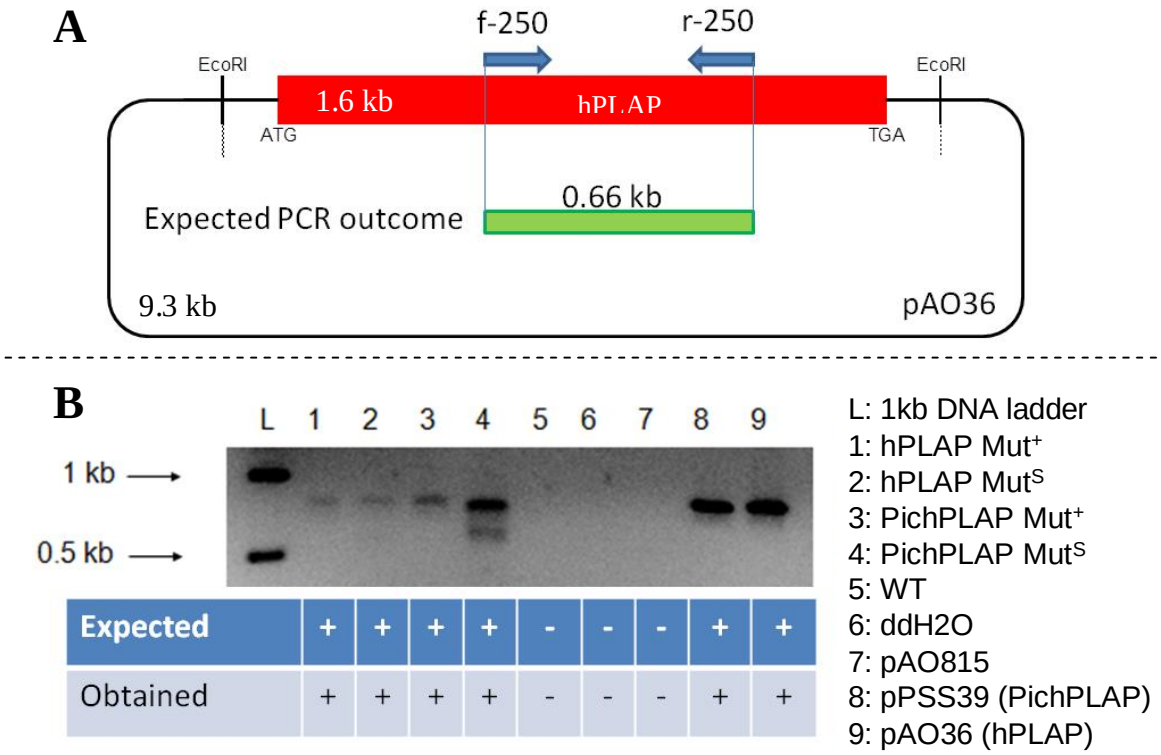


Figure 4.15: PLAP presence confirmation by PCR
A: The primers f-250 and r-1000 should amplify by PCR a 0.66 kb fragment if the PLAP gene is present in a given DNA sample (i.e.). B: This electrophoresis gel showed the 0.66 kb fragments amplified by PCR to confirm plasmid PLAP presence in all the new *Pichia pastoris* GS115 strains (positions 1-4). The plasmids used for the transformation and creation of the new strains were used as positive controls (positions 8-9). Positions 5-7 have been used as negative controls for PLAP presence. The table resumes the results obtained and shows that all results were as expected, and, therefore, PLAP integration into the *Pichia pastoris'* genome was successful.

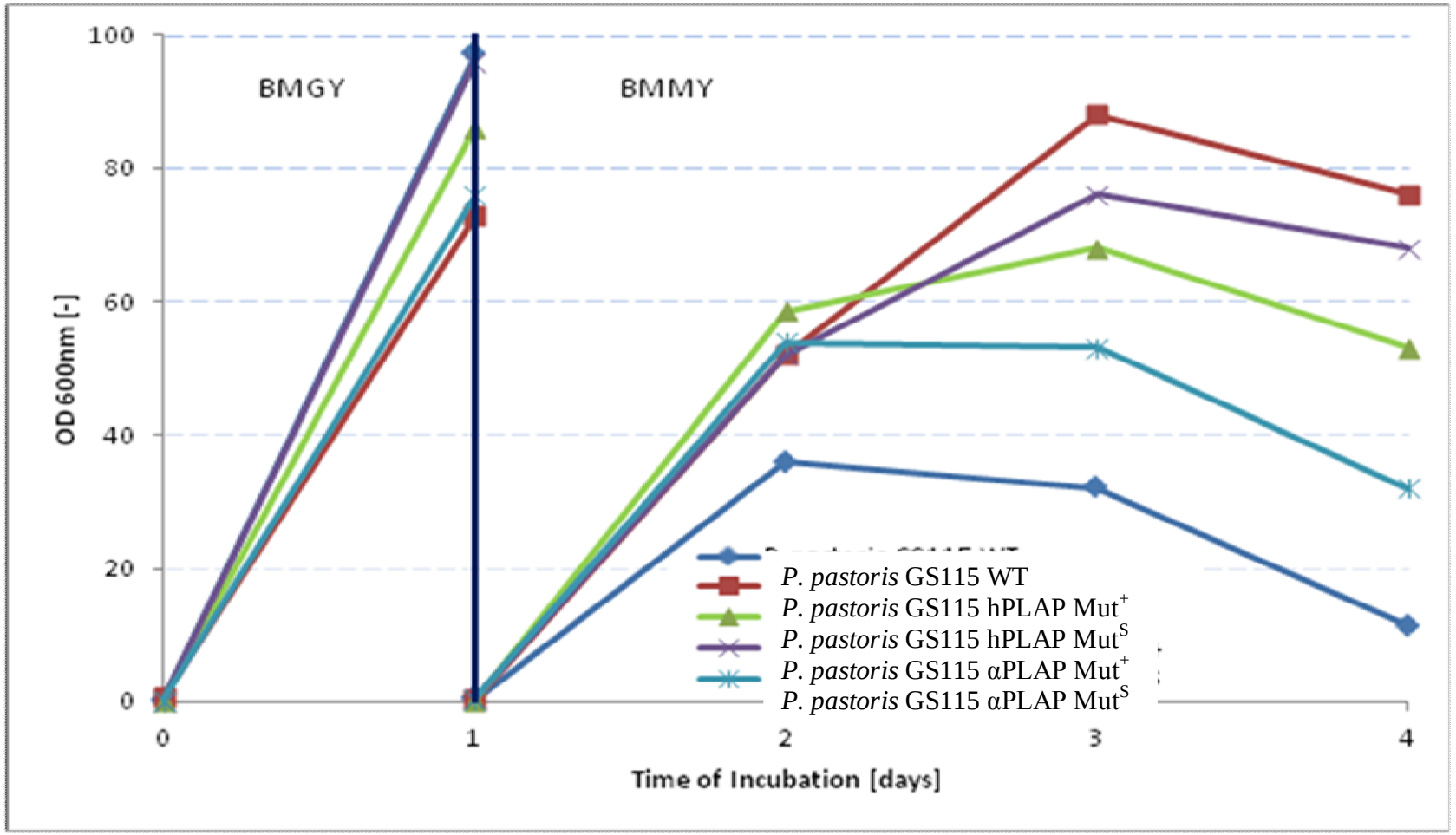


Figure 4.16: Growth curve of different *Pichia pastoris* GS115 strains in shake flasks
Cells were grown in Glycerol (BMGY) for the first day (24 hours), then transferred to methanol-containing media (BMMY) at an $OD_{600} \approx 1$ for the rest of the cultivation. Methanol was added every 24 hours at a final concentration of 0.5% and it was increased by 0.5% at every addition up to 2% as follows: inoculation - addition of 0.5% methanol, 24 hours – 1% methanol, 48 hours - 1.5% methanol, 72 hours – 2% methanol. Methanol % is calculated on the total volume. At every addition it is supposed that all previous methanol has been completely consumed.

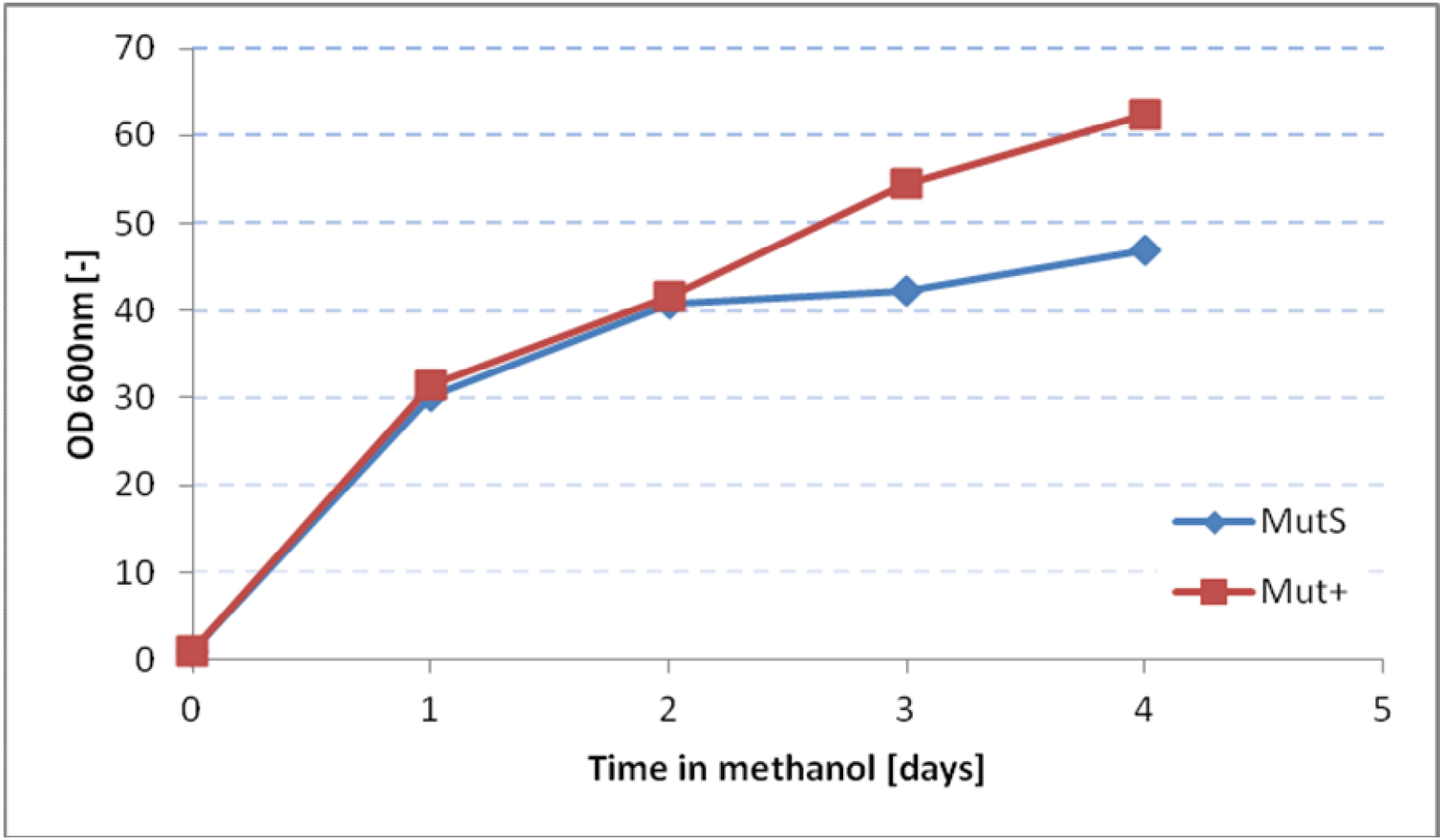


Figure 4.17: Growth curve of *Pichia pastoris* GS115 hPLAP Mut$^{+/S}$ strains in shake flasks
Cells were grown in Glycerol (BMGY) for the first 24 hours (results not showed), then transferred to methanol-containing media (BMMY) at an $OD_{600} \approx 1$ for the rest of the cultivation. Methanol was added every 24 hours at a final concentration of 0.5%.

4.4. Conclusions

In summary, new *Pichia pastoris* strains were successfully created (Table 4.6). The PLAP gene sequence was confirmed not to have a mutation. Similarly, genomic insertion via homologous recombination was also confirmed. Growth tests on Petri dished established that both Mut^+ and Mut^S phenotypes grew as expected. Finally, it was shown in shake flasks that cells are able to grow in methanol concentrations exceeding the conventional 0.5% (v/v) value.

Table 4.6 Created *Pichia pastoris* strains
New *Pichia pastoris* strains created as part of this project.

Strain	**PLAP signal**	**Vector used for transformation**
Pichia pastoris GS115 Mut^+ HIS4 hPLAP	h	pAO36
Pichia pastoris GS115 Mut^S HIS4 hPLAP	h	pAO36
Pichia pastoris GS115 Mut^+ HIS4 αPLAP	α	pPSS
Pichia pastoris GS115 Mut^S HIS4 αPLAP	α	pPSS

5. Effect of high methanol feed rates on *Pichia pastoris* biomass accumulation in bioreactor cultivation

The first step for *Pichia pastoris* cultivation optimisation was a biomass production study. This was done by increasing the suggested maximal methanol feed (10.9 ml/hour/L initial fermentation volume) in the conventional protocol by 80 to 100%. In addition to the methanol feed, other parameter have been tested for an effect on biomass formation. Those parameters were initial post-inoculum fermentation optical density (OD), scale (1 or 20 litres vessel), induction mode (step induction or direct induction), presence/absence of a transition phase (starvation) between the glycerol feed and the methanol induction, and the lengths of the starvation and methanol induction phases. Of those some variations have been recorded during growth. However, in all cases the final outcome was similar (OD values of about 1000 units), and therefore they will not be presented in this work.

5.1. Fermentation protocol modifications

Fermentations for biomass optimisation were primarily performed in an Applikon 20L vessel. During growth optimisation initial fermentation conditions were investigated: optical density of inoculum, pH 6.5 (adjusted with ammonia 25% (v/v) and phosphoric acid 30% (v/v)), dissolved oxygen tension (DOT), and air feed rate. During fermentation 40% oxygen and pure oxygen were used as required. Glycerol fed-batch started after 17.75 hours with a feed of 17.47 ml/h/L of initial fermentation volume instead of 18.15 ml/h/L. A 30 minutes starvation was introduced at the end of the glycerol fed-batch.

During the methanol fed-batch phase once reached the maximal feed (10.9 ml/hour/L initial fermentation volume) suggested in the conventional protocol, this one was increased overtime to reach between 21.0 and 37.5 ml/h per litre of initial fermentation volume depending on cell's behaviour. The methanol was increased according to the DOT probe reading of the fermenter. DOT spikes and/or DOT increases were followed by a flow rate increase. Air flow rate during methanol fed-batch phase was kept constant with cascade control followed by 40% oxygen and pure oxygen as required. All experiments at 1 litre scale were performed in the same way, but instead of using 40% oxygen before starting with pure oxygen, the reactor's built in oxygen blending system was used. After the impeller reached maximal speed, oxygen blending was set to automatic mode in order to maintain DO levels at about 30%.

5.2. Fermentation biomass optimisation

As described in the introduction chapter some work on increased methanol feed rate aimed to increase biomass formation has already been done. However, to date little evidence was found on the improvement of protein production at higher biomass. The goal was to investigate the feasibility of initially quickly increasing biomass with high methanol feed rate, and then reduce the methanol feed in order to increase total protein yield (Trihn *et al.*, 2003).

First it was done an evaluation of the effect of increased methanol feed (High Methanol Method - HFM) in *Pichia pastoris* GS115 Mut$^+$ HIS4 hPLAP cells (Figure 5.1) compared to the conventional method (CFM) at 20 litres scale. Results, showed that

HFM achieves similar biomass to CFM, but in less time at both 1 and 20 litres scales (Figures 5.1 and 5.2). Reproducibility studies at both scales showed that growth curves with both methods were consistent at both 1 and 20 litres scales (Figures 5.3 and 5.4). In addition, it seems that the induction method (step increase slow or fast) does not have influence of the biomass generation outcome (Figure 5.3 and 5.4). Jahic *et al.* (2002) showed a 14% increase in biomass with a 50% higher methanol feed. Similar results were obtained with a 50% higher methanol flow-rate 12 hours post induction (Figure 5.1) at 20 litres scale. However, at 20 litres scale an almost doubled feed 19.5 mL/h/L in HFM - compared to CFM (10.9 mL/h/L) - increased biomass formation after 24 hours in methanol by about 100% (Figure 5.1). At 1 litre scale results were similar, but after 24 hours in methanol the biomass increase was slightly lower (about 40%) compared to the 20 litres scale (Figure 5.2).

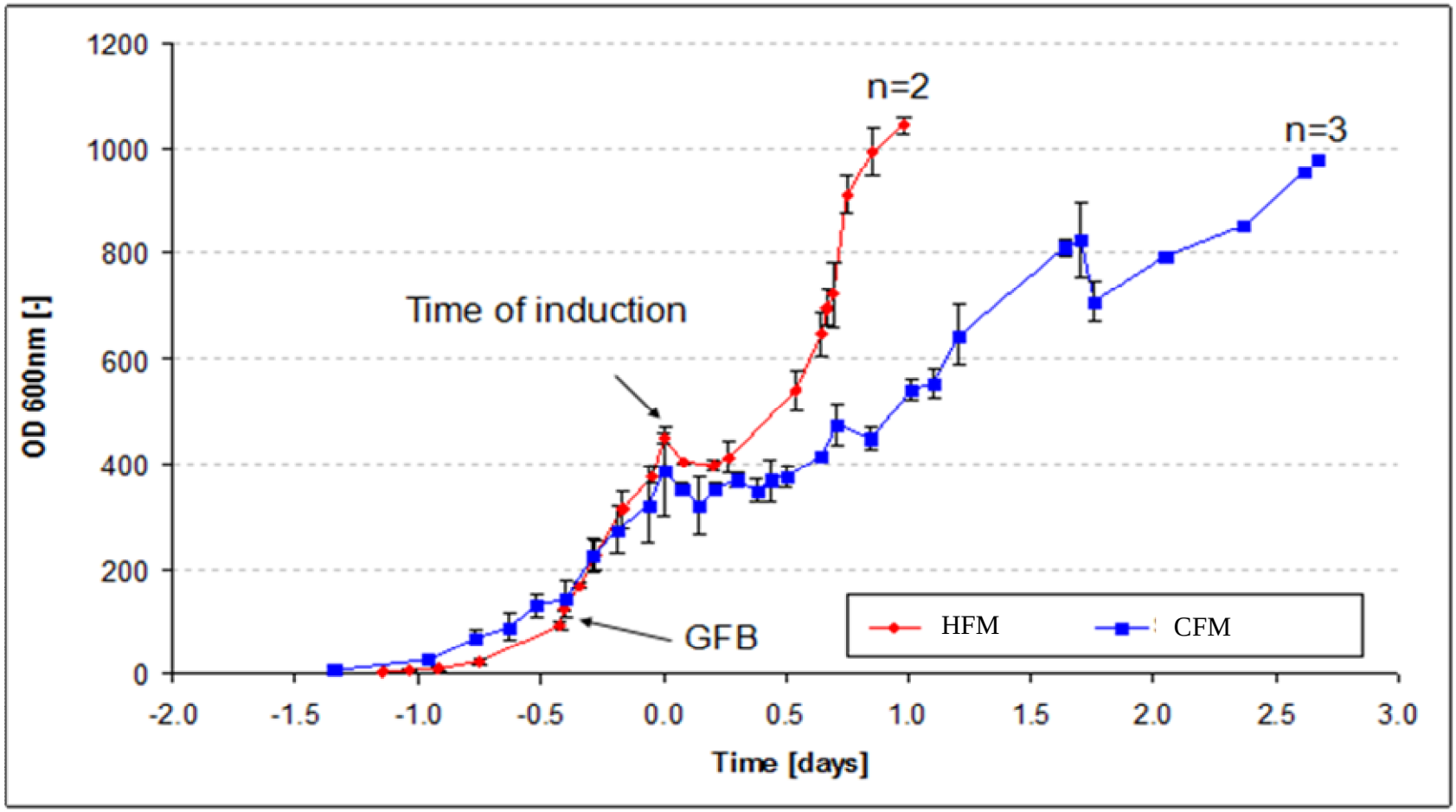


Figure 5.1: HFM vs. CFM growth curves at 20L scale
Growth curve of *Pichia pastoris* GS115 hPLAP Mut$^+$ strains at 20L scale. Cells are first grown in Glycerol, then they are adapted to methanol (induction) and grown using two different methods (conventional methanol feed method and high methanol feed method). Results show similar biomass formation in less time for HFM. Error bars are generated from OD readings of different fermentations. GFB: Glycerol fed-batch.

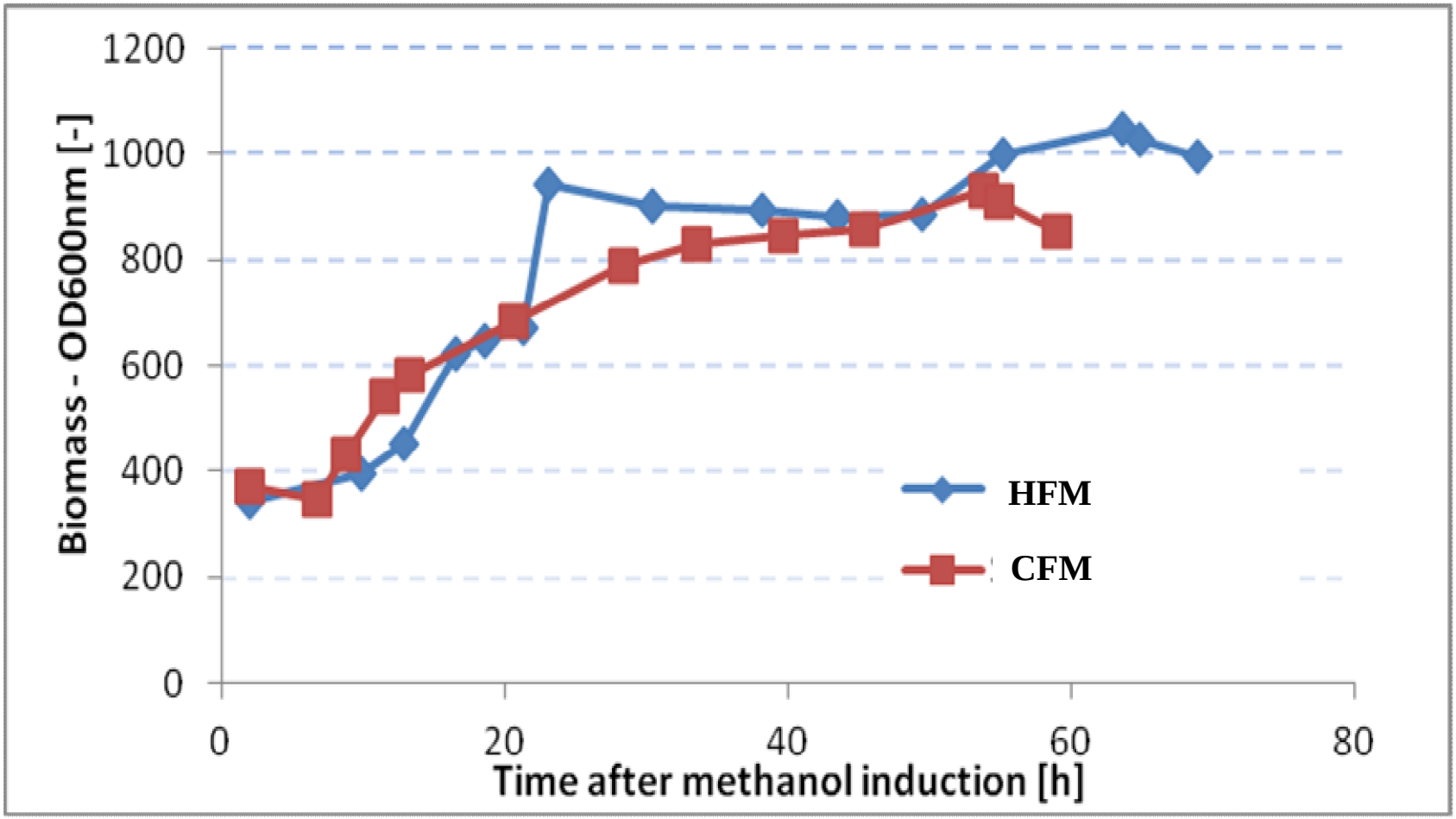


Figure 5.2: HFM vs. CFM growth curves at 1L scale

Growth curve of *Pichia pastoris* GS115 hPLAP Mut^{+} strains at 1L scale. Cells are first grown in Glycerol, then they are adapted to methanol (induction) and grown using two different methods (conventional methanol feed method and high methanol feed method). Results show similar biomass formation in less time for HFM (after 24 hours post-induction). Time 0 is the time of methanol induction. In this figure n was equal 1. The fermentation method was the same as 20 litres scale.

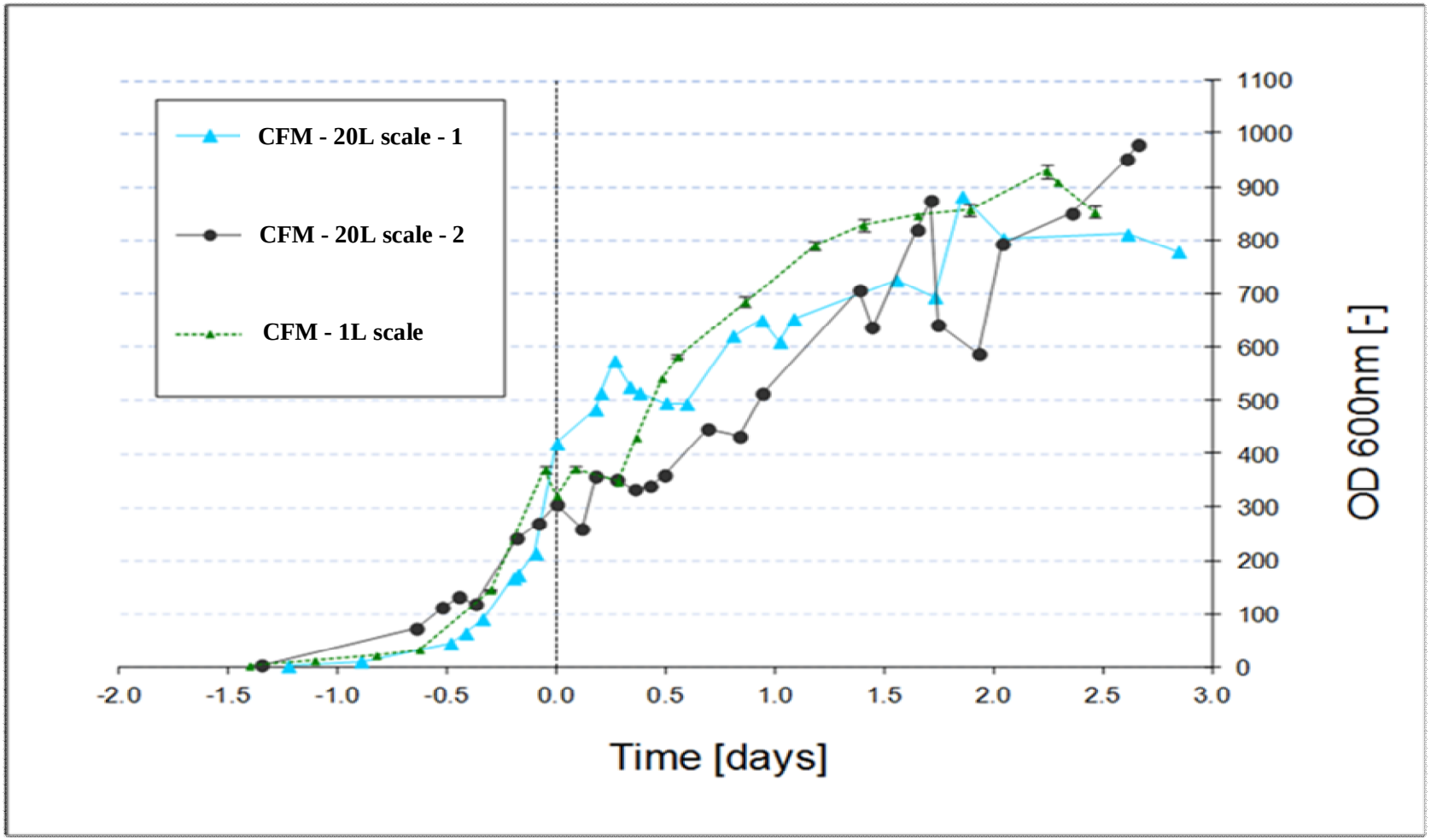


Figure 5.3: CFM growth curve of *Pichia pastoris* GS115 hPLAP Mut$^+$ strains at 1 and 20L scales
Cells are first grown in Glycerol, then they are adapted to methanol (induction) and grown using the conventional feed method. Results show that biomass performance for CFM is consistent at 1L and 20L scale. Time 0.0 is the time of induction.

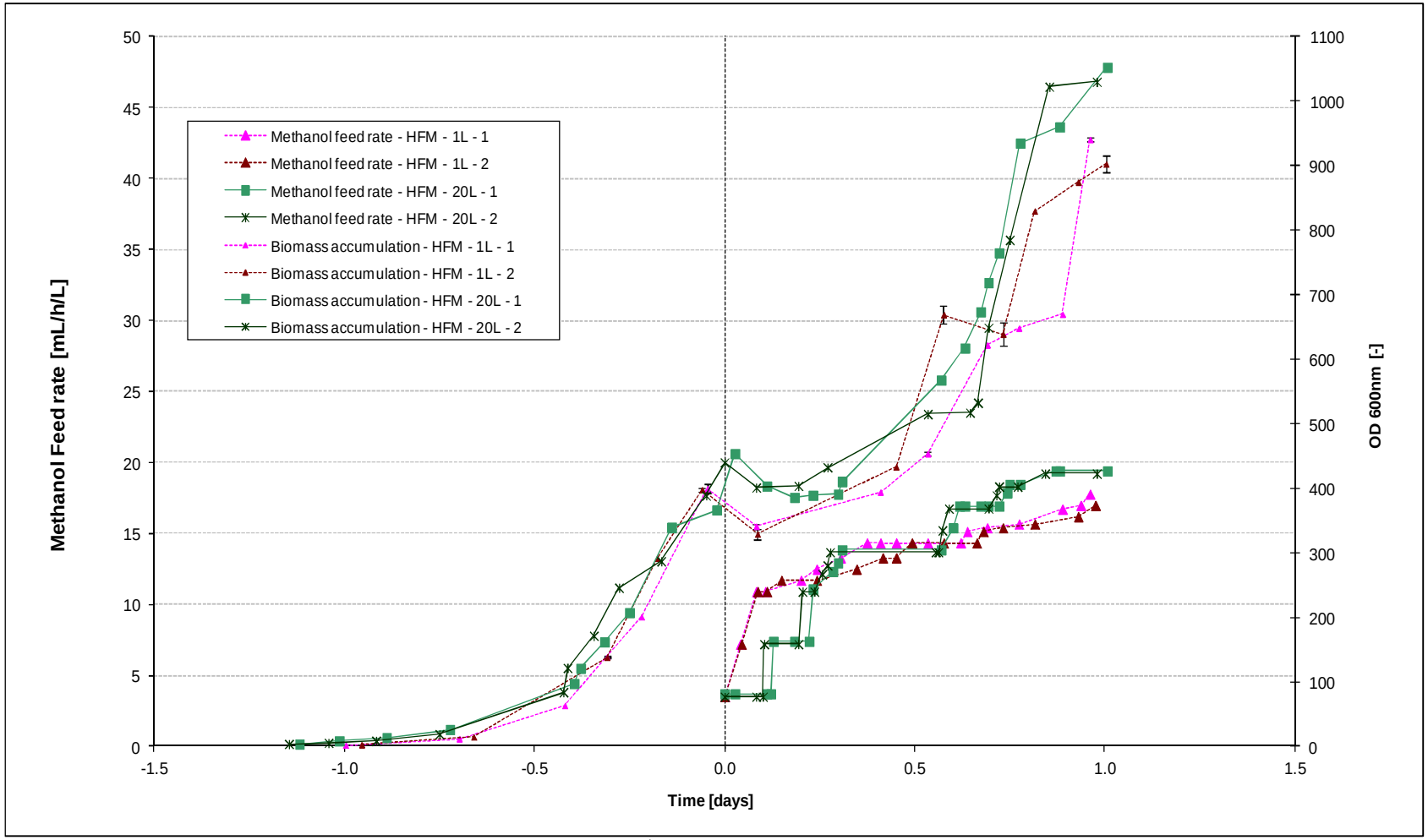


Figure 5.4: HFM growth curve of *Pichia pastoris* GS115 hPLAP Mut$^+$ strains at 1 and 20L scales
Cells are first grown in Glycerol, then they are adapted to methanol (induction) and grown using the high methanol feed method. Results show that biomass performance for HFM is consistent at 1L and 20L scale, even with different feeding profiles. Time 0.0 is the time of induction.

5.3. Conclusions

It was demonstrated that it is possible to boost biomass formation in a *P. pastoris* cultivation to OD of over 1000 units. In addition, by feeding higher (up to 80-100%) methanol concentrations to the cells, they are able to grow to over 40% higher (after 24 hours post-induction) than the OD typically achieved by CFM and 50% higher than that reported by Jahic *et al*. (2002). It also appeared that the induction method had not or little effect on biomass generation in the methanol phase and that growth curves had high reproducibility, also by scaling from 1 to 20 litres culture.

6. Effect of high methanol feed rate on *Pichia pastoris* recombinant protein production during bioreactor cultivation

To increase productivity per cell, and therefore the volumetric and specific yield of a model protein (PLAP) a high methanol feed methods was compared to the conventional methanol feed method

This chapter was divided in three main sections. First, a shake flasks study aimed to screen several *Pichia pastoris* strains for PLAP production in high methanol-containing media was performed. Then, the high methanol method (HFM) described in Chapter 5 was tested for protein production levels and compared to the conventional methanol feed method (CFM) at different scales (1 and 20 litres). The last section of this chapter, however, was aimed to describe a novel method, in which HFM and CFM were combined in a single protocol, to increase cell's productivity and volumetric yield.

6.1. Strains comparison at shake flask level

Four different strains of *Pichia pastoris* GS115 $Mut^{+/S}$ have been created in the frame of this project having two different signal peptides: hPLAP and αPLAP. Before proceeding with bioreactor cultivation, some preliminary tests at shake flasks level have been performed in order to identify the best performing strains in terms of total protein (PLAP) production.

In this work the PLAP concentration has been measured as the total amount (see material and methods section). In fact, preliminary measurements showed low to no secretion for the created strains. On the other hand, it has been noticed that PLAP was

accumulated within the cell. As a consequence, all the PLAP data shown in this work is the result of the sum of secreted and retained PLAP. It was not part of this work investigating reasons for poor performance of the secretion signals used, nor trying to optimise secretion levels.

Tests indicated that among the Mut^+ strains, hPLAP had potentially the highest protein production (all PLAP assay readings in shake flasks have been performed after two days incubation in methanol (BMMY) at an OD_{600nm} of about 50 [-]). This strain has then been used for preliminary bioreactor experiments. The other strains (including Mut^S) were supposed to be tested in bioreactor as well, but those tests have been cancelled. This decision was made before PLAP assay data were available for both strains as it was observed in Mut^+ hPLAP clones that productivity was low.

From Figure 6.1 below it appeared that Mut^S strains had higher volumetric yield than Mut^+ strains. Interestingly, for Mut^S strains, αPLAP clones showed a slightly higher PLAP concentration compared to hPLAP clones. Two strains have been used as controls: *Pichia pastoris* GS115 Mut^+ wild-type (WT) and *Pichia pastoris* GS115 Mut^S Human Serum Albumin (HSA). In fact, they should only express minimal endogenous alkaline phosphatase, and, as expected, the assay confirmed minimal combined alkaline phosphatase activity in these strains.

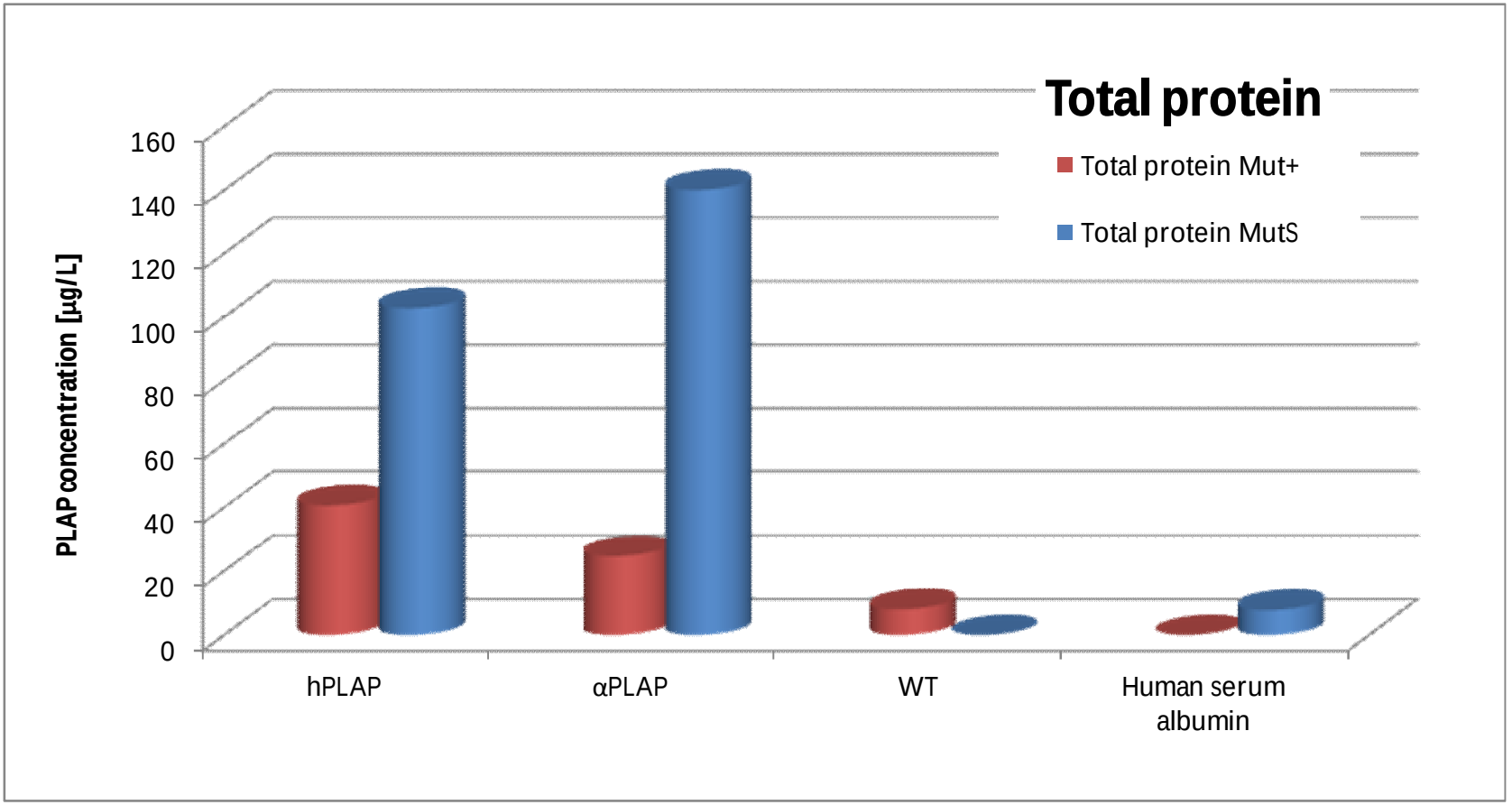


Figure 6.1: Shake flasks PLAP Quanti-blue™ colorimetric assay
PLAP Quanti-blue™ colorimetric assay for different *Pichia pastoris* Mut$^{+/S}$ strains after 2 days shake flask incubation in methanol at an OD of about 50. The PLAP concentrations expressed are the sum of secreted and non-secreted PLAP. Readings are taken in a 96-wells plate reader. Mut^{+} hPLAP strain has the best performance at shake flask level among other Mut^{+} strains. However, Mut^{S} strains show higher product titres compared to Mut^{+}, where the αPLAP clone is the best performer. Two strains have been used as controls: *Pichia pastoris* GS115 Mut^{+} wild-type (WT) and *Pichia pastoris* GS115 Mut^{S} Human Serum Albumin (HSA).

Interestingly, the chemiluminescent assay, in contrast to the colorimetric assay, showed in one of the readings higher productivity (over the double) for *P. pastoris* GS115 Mut^+ αPLAP than *P. pastoris* GS115 Mut^+ hPLAP (Figure 6.2). However, as stated above, this assay had very low reproducibility with high degree of variance, and therefore this measure has not been taken into consideration. Even though this assay (chemiluminescent) seemed to generate a better protein production profile for Mut^+ strains at shake flasks level (results not shown) compared to the colorimetric assay (Figure 6.1), its difficult optimisation (more details in chapter 3) made it impractical to be used in the frame of this project.

Data found in the literature is somehow discordant as productivity is usually product dependent. In fact, there are papers stating that Mut^+ strains have higher production potential, and others stating that Mut^S clones can produce higher recombinant protein titres. Krainer *et al.* (2012), for instance, showed an higher specific substrate uptake rate for Mut^+ strains, yet higher specific HRP and volumetric productivities in Mut^S clones. Kim *et al.* (2009) showed that productivity increased during fermentation up to 6-fold going from shake flask to 5L culture.

As described in previous sections, all relevant PLAP data shown in this work was expressed as total combined PLAP rather than secreted PLAP. In fact, secreted PLAP activity was near to zero. On the other hand, non-secreted PLAP concentrations per mL of culture broth seemed to be higher. Therefore, the decision of expressing all the results as the sum of secreted and retained PLAP was made. These data have been confirmed at both shake flask and bioreactor levels. Since the objective of this work was to optimise

growth to maximise production, no further tests have been performed to explain or understand this poor secretion performance of the created strains.

6.2. Methanol feed rate affects at high cell density

After biomass studies, the effect of both methods (high methanol feed rate method (HFM) and conventional feed rate method (CFM)) on protein production at 1 and 20 litres scales was investigated. For both methods (Figure 6.2) the scale did not influence protein production, indicating ease of scalability of both protocols. The CFM did offer greater volumetric yield compared to HFM. After 50 and 25 hours from methanol induction, the 20 litres fermentation was stopped for both CFM and HFM, respectively. Those cultures were stopped as the maximal working volume (15 litres) within the vessel was reached. There were two options to continue the fermentation for longer time: starting with lower initial volume or remove material after fermentation started. However, neither of them was practical. Starting with less material was impossible because of the geometric conformation of the reactor, in which the lowest impeller was already barely submerged by the media. Therefore starting with less material would have led to non-mixing conditions in the first part of fermentation (glycerol batch). On the other hand, removing material (partial harvesting) and continuing the fermentation could have been an option to augment fermentation length. However, this solution was not put into practice as DO readings became erratic after removing 2 litres culture broth and of difficult stabilisation due to the use of pure oxygen (oxygen blending was not available at that scale). These oxygen variations lead to a substantial cell death (about 15%, tested

with Trypan Blue 0.4% stain), and therefore, the results obtained by continuing the fermentation would have not been representative, and comparable to the ones from the 1 litre scale. Another problem caused by the erratic DO readings was that this value fell under the limit of 15%. For instance, Trentmann *et al.* (2004) stated that below 15% oxygen saturation, no or low product accumulation was recorded. As a consequence, the decision of stopping the culture was taken. At industrial level, HFM method for heterologous protein production could be adjusted by adding an extra impeller at the bottom of the bioreactor, allowing to reduce the initial fermentation volume and therefore allowing longer fermentation times. However, if the objective is purely to generate biomass this procedure is not required and material could be harvested within the first 24-48 hours after methanol induction.

The 1 litre fermentations, on the other hand, were stopped after about 60 to 70 hours post-induction. In fact, most papers reported their highest volumetric protein yield after 72 hours from induction, with total fermentation length up to 140 hours. Data from Figure 6.3 for HFM indicated that when cells are forced to grow under augmented methanol feed rate, they produce little recombinant protein (as expected). However, data from CFM (Figure 6.2) indicated an higher and exponential protein production starting from about 20 hours post methanol induction. In this case the fermentation was stopped after 60 hours from methanol induction because of technical fault of the vessel. By looking at the volumetric yield trend from CFM at 1 litre scale it is hypothetically possible to estimate a potential protein production matching or overtaking literature values. In actual facts, Heimo *et al.* (1998) and Chen *et al.* (2004) recorded PLAP concentrations of about 2 and 8 mg/L, respectively. By looking at volumetric yield

profiles obtained, protein concentrations of about 10 to 40-fold lower, were recorded at 1 litre scale using the same method (CFM – Figure 6.2). However, it has to be said that literature values were recorded for secreted PLAP only, whereas results in this work are expressed as total PLAP (secreted and retained), since it was found that secretion levels were near to zero in all samples, whereas retained PLAP activity was higher for all tested samples in this work.

By looking at HFM, poor PLAP activity was recorded. Apparently, forcing cells to grow in higher methanol drove all or most of the energy in the cell towards biomass generation rather than product formation, and as a consequence, lower product yields were recorded. Trentmann *et al.* (2004) observed that increased methanol uptake boosted growth but not recombinant protein production and that maximal productivity in Mut^+ strains was obtained at approximately one-third of maximum growth rate. However, this phenomenon is protein specific and some variations could be recorded (Zhang *et al*., 2000). Jahic, *et al*. (2002) also observed this phenomenon of reduced productivity and higher biomass accumulation with increased methanol feed rate. Thus, increasing methanol feed increased cell growth rate and inhibited product formation (Trihn *et al*., 2003).

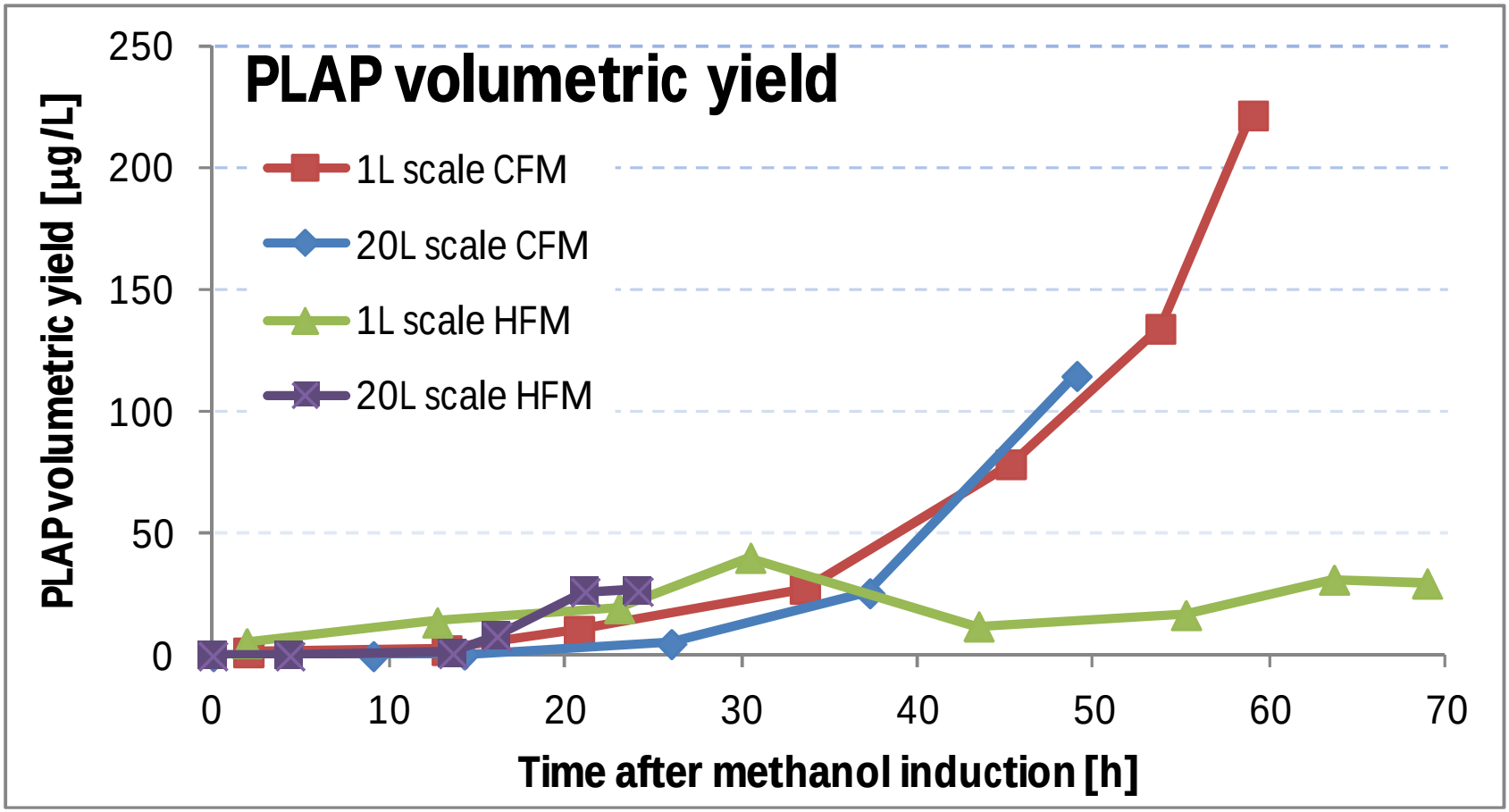


Figure 6.2: PLAP volumetric yield scale comparison for CFM and HFM
Curves generated from the fermentation of *Pichia pastoris* GS115 hPLAP Mut^{+} strains at 1 and 20L scales using the conventional feed method (CFM) and high methanol feed method (HFM). Time 0.0 was the time of induction. **CFM:** Volumetric yield was comparable at both scales for up to 50 hours after methanol induction. After that time, the 20 litres fermentation was stopped as maximal working volume was reached within the vessel. **HFM:** Volumetric yield is comparable at both scales until 25 hours after methanol induction. Fermentation at 20L scale was stopped after about 25 hours in methanol because maximal working volume in the bioreactor was reached during methanol fed-batch phase. Values after 24 hours from induction for the 1L scale are derived from the combination HFM/CFM. The PLAP concentrations expressed are the sum of secreted and non-secreted PLAP

As described above, HFM offered better biomass generation and CFM offered better protein production (Figures 5.3-5.4 and 6.2). Data from Figures 6.3 and 6.4 clearly outlined higher total protein production in CFM. The methanol pump profiles indicated that an about 80-100% higher feed-rate did not increase protein production and further confirmed that the cell metabolism prioritised growth rather than production. In terms of total protein (Bradford assay), a substantial increase was recorded for both methods after methanol induction due to the methanol utilisation pathway activation. Final concentrations reached about 2.5 g/L total protein, indicating for CFM that PLAP was only about 0.01% of the total cell's protein production (results not shown).

.

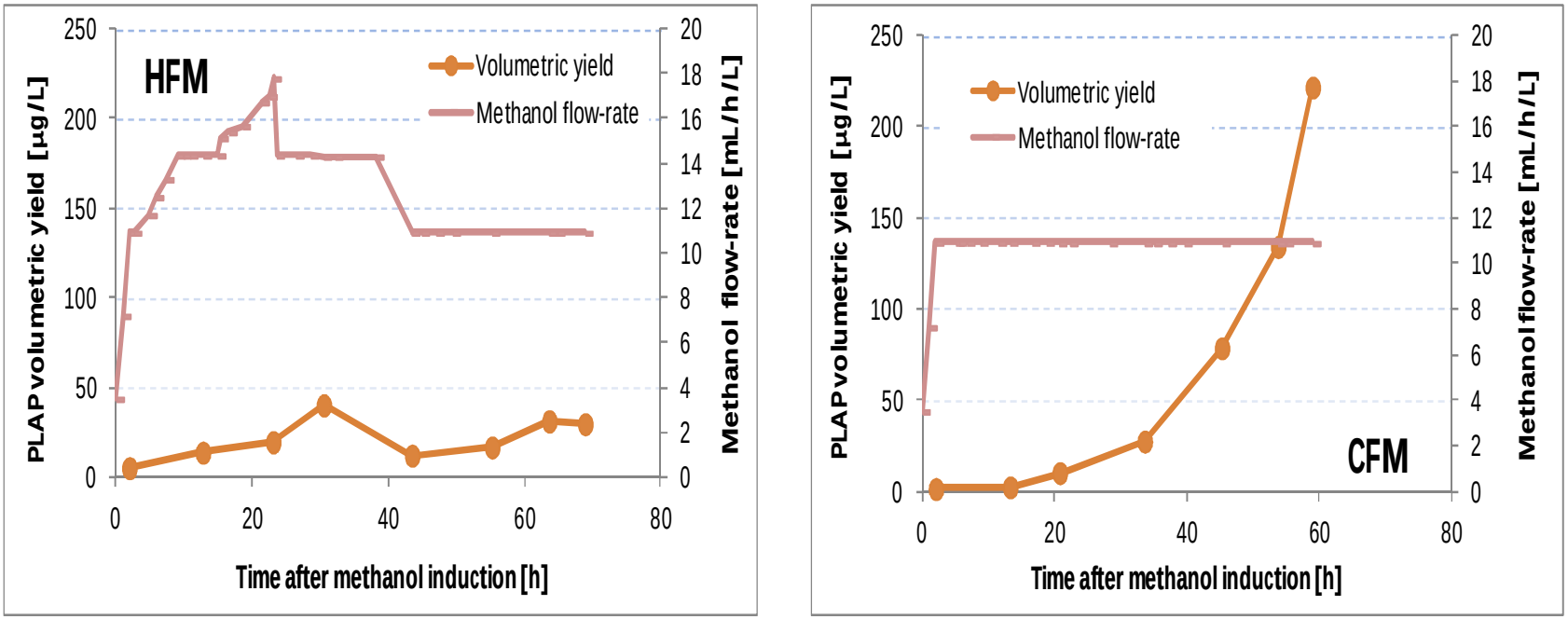


Figure 6.3: PLAP volumetric yield method comparison at 1L scale
Curves generated from the fermentation of *Pichia pastoris* GS115 hPLAP Mut$^+$ strains at 1L scale using HFM and CFM methods. The methanol feed rate was reduced in HFM after about 25 hours post-induction to generate the combination method HFM/CFM. Up to that time the volumetric yield was slightly higher for HFM. At the end of fermentation, however, PLAP volumetric yield was significantly higher in CFM than in HFM/CFM. Time 0.0 was the time of induction. The PLAP concentrations expressed are the sum of secreted and non-secreted PLAP

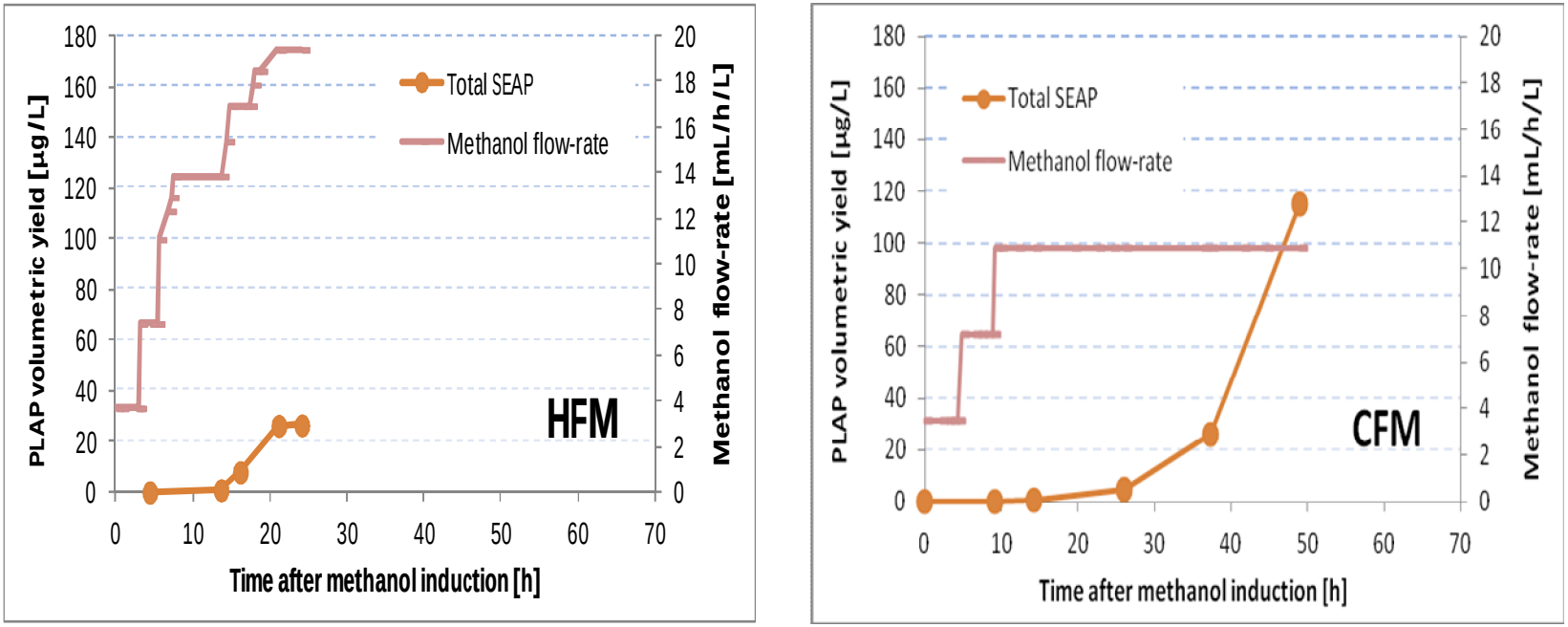


Figure 6.4: PLAP volumetric yield method comparison at 20L scale
Curves generated from the fermentation of *Pichia pastoris* GS115 hPLAP Mut^{+} strains at 20L scale using HFM and CFM methods. The methanol feed rate was stopped in HFM after about 25 hours post-induction because the maximal working volume in the bioreactor was reached. Up to that time the volumetric yield was slightly higher for HFM. Time 0.0 was the time of induction. The PLAP concentrations expressed are the sum of secreted and non-secreted PLAP

6.3. Conventional Methanol Feed Rate Method (CFM) and High Methanol Feed Method (HFM) combined

As previously described, methanol feed-rate had an important impact on the production of recombinant PLAP in *Pichia pastoris* GS115 Mut^+ host cells. Higher methanol supply increased biomass generation. Similarly, also protein production was increased after about 24 hours post methanol induction compared to the conventional feeding method. By looking at the single methods alone lower protein concentration compared to literature values were recorded. However, fermentations had to be stopped due to volume issues, and it was possible, in projection, to match literature values with longer fermentation times. The next step was, therefore, to combine the two methods (HFM and CFM) in a single one in order to first quickly generate high biomass (HFM) and then increasing protein production (CFM) by lowering the methanol feed-rate (Figure 6.5), with the aim to match or overtake the literature values. Two different approaches were used to reduce the methanol feed rate: drastic and progressive. The drastic approach consisted in reducing the methanol feed to match the one of CFM (10.9 ml/h/l). Conversely, the progressive method had one extra step of flow reduction before reaching the CFM feed value. For both methods, the DO reading was kept constant by adapting the oxygen supply while changing the methanol feed. Figure 6.5 shows that while the progressive method offered a smooth adaptation of the cells to the new conditions, the drastic method had an adaptation step in which the methanol flow rate had to be stopped and restarted to avoid accumulation and consequent cells damage. Additionally, this figure clearly shows that once the methanol feed was reduced, cells did stop their growth,

which restarted later with slower growth rate. Final OD values were comparable with a delay of over 24 hours to reach an OD of about 1’000 units.

The main objective of this project was to create a new standard method aimed to maximise protein production. Even with lower-than-literature values, but higher than single methods alone, this methods would have been promising for improved protein production. However, this was not the case. In fact, Figure 6.6 for HFM at 1 litre scale was a combination of phases of the two methods, where the first 24 hours in methanol were done with HFM, and after that time methanol flow rate was reduced to follow CFM. On the other hand, a failed fermentation using the HFM/CFM combination (Figure 6.7) showed a comparable level of PLAP compared to CFM. Therefore, it was possible that some other parameter played a role in the protein production machinery. In fact, the methanol feed was started later compared to other HFM, allowing longer starvation. This is because the temperature control failed and temperature rose to almost 40°C. Ling *et al.* (2010), showed, in shake flasks, that temperature can rise by up to 25°C if left uncontrolled. They also stated that by reducing the culture temperature during induction proteolytic degradation of recombinant proteins reduces. In addition, cell viability and product yield are increased by lowering the temperature to 23°C.

In this case cells did not die and they had a later quick adaption to methanol, that surprisingly did not boost biomass formation (remained steady with only a little step increase), but did boost protein production instead. Interestingly, during the step increase in biomass, product formation dropped, but restarted after biomass stabilised once more due to another temperature jump. Production formation kept rising also during steady methanol feed (CFM), but finally dropped after a last temperature jump (after about 50 hours in methanol) resulting in cell death and consequent fermentation termination. In conclusion, it appeared that by stressing the cells with temperature jumps during methanol induction and subsequent HFM, energy that would be driven to biomass generation, was instead used by cells for protein production as CFM volumetric yield was matched.

Although further tests on this subject were not performed it would be interesting investigating further the combination of two different methods on a single cultivation. In addition, cultivations for the two methods combination were started with already low performing strains (in terms of protein production). As a future project, it would be therefore interesting to create a new strain (or to use a commercially available one) which already exhibits a similar-to-literature protein production and test the combination of the two methods once more.

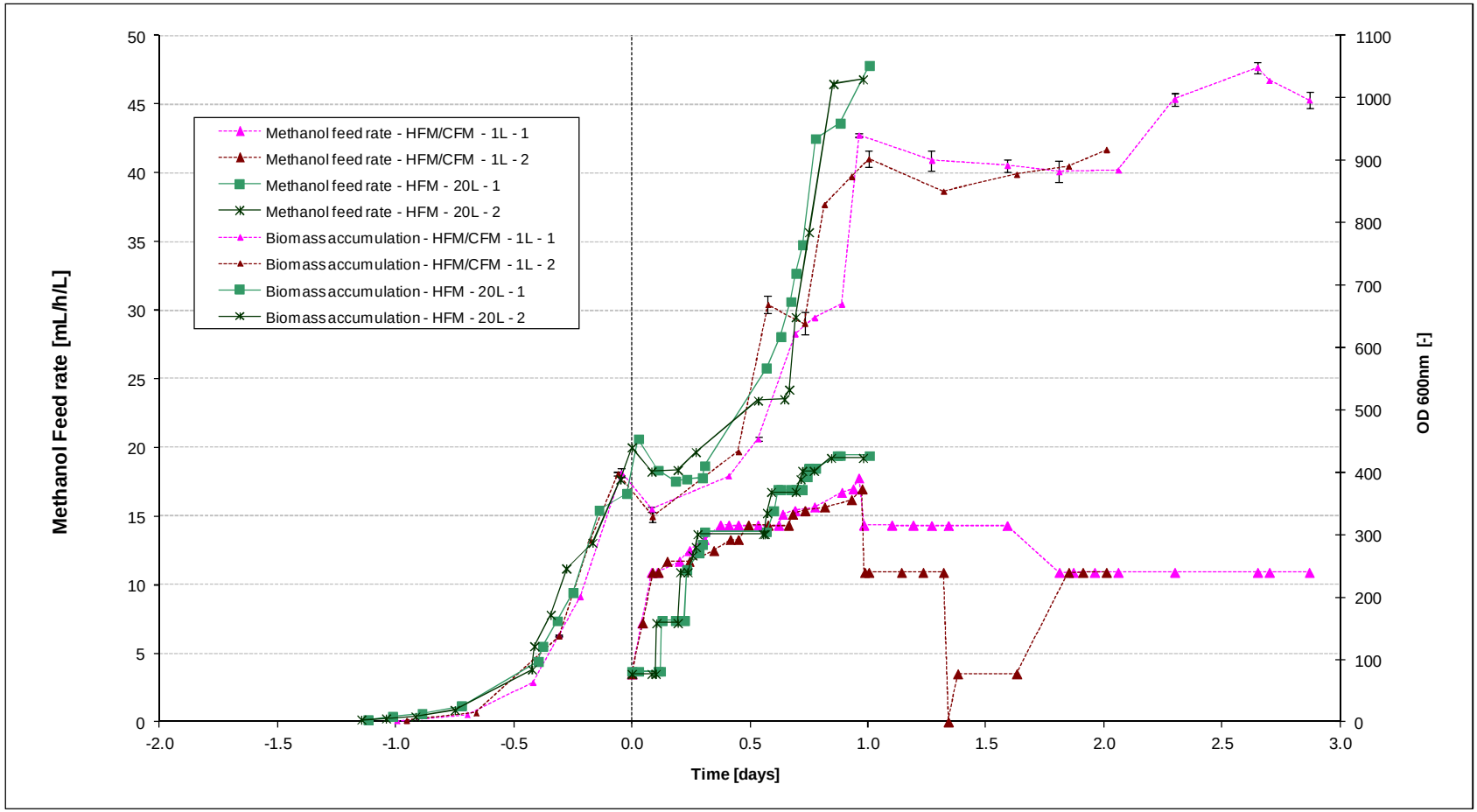


Figure 6.5: Biomass profiles at 1 and 20L scales for HFM and HFM/CFM, with methanol feed profiles
Curves generated from the fermentation of *Pichia pastoris* GS115 hPLAP Mut^{+} strains. Biomass profiles at both scales were comparable during glycerol batch and fed-batch phases. They were also comparable until about 24 hours in methanol, when HFM alone cultivation was stopped and methanol flow rate was reduced for HFM/CFM combination.

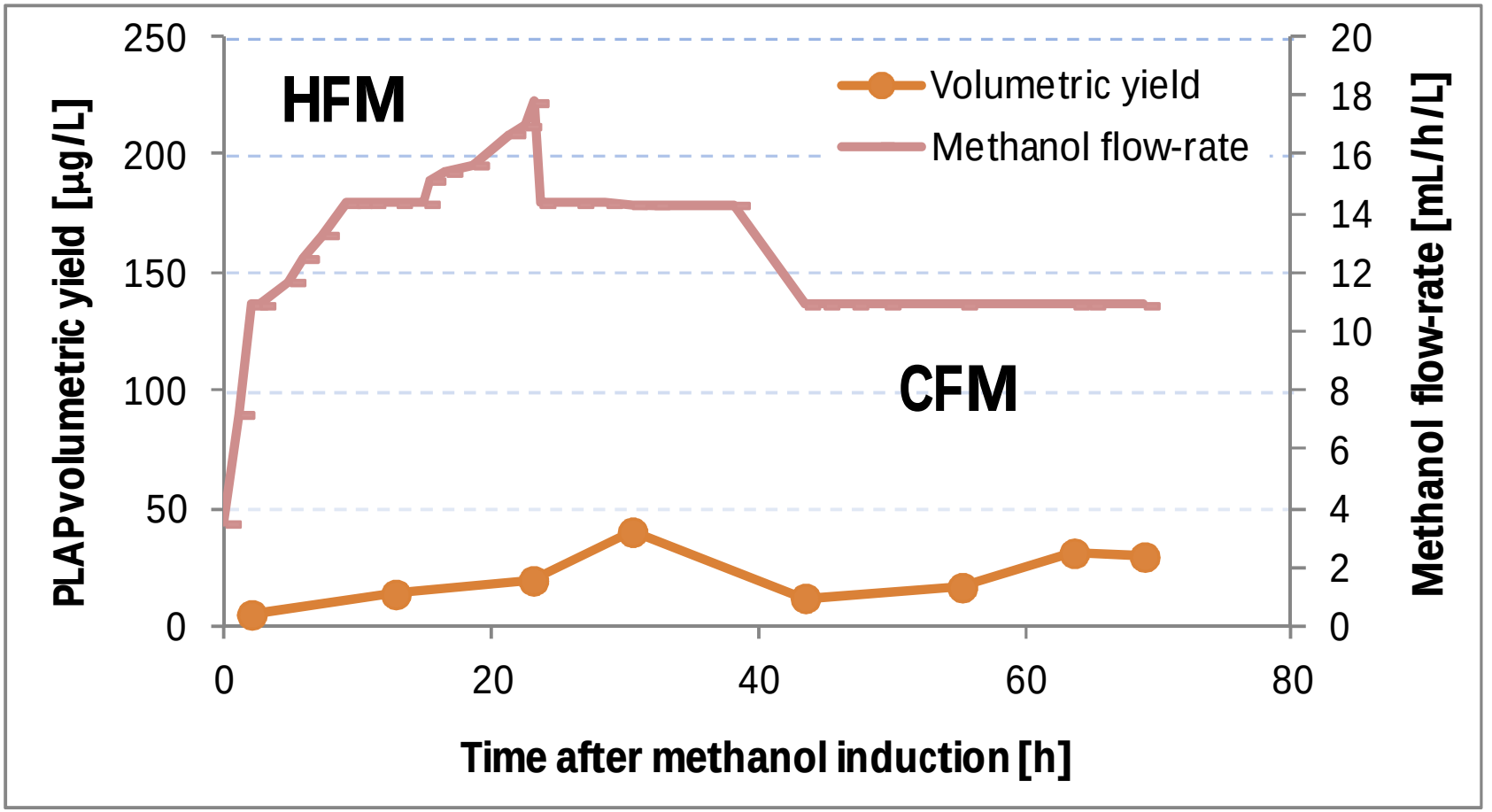


Figure 6.6: PLAP volumetric yield combined method at 1L scale
Curves generated from the fermentation of *Pichia pastoris* GS115 hPLAP Mut$^+$ at 1L scale using HFM/CFM combination. The methanol feed rate was reduced in HFM after about 25 hours post-induction to generate the combination method HFM/CFM. At the end of fermentation, however, PLAP volumetric yield did not rose to levels higher than before method change. Time 0.0 was the time of induction. The PLAP concentrations expressed are the sum of secreted and non-secreted PLAP.

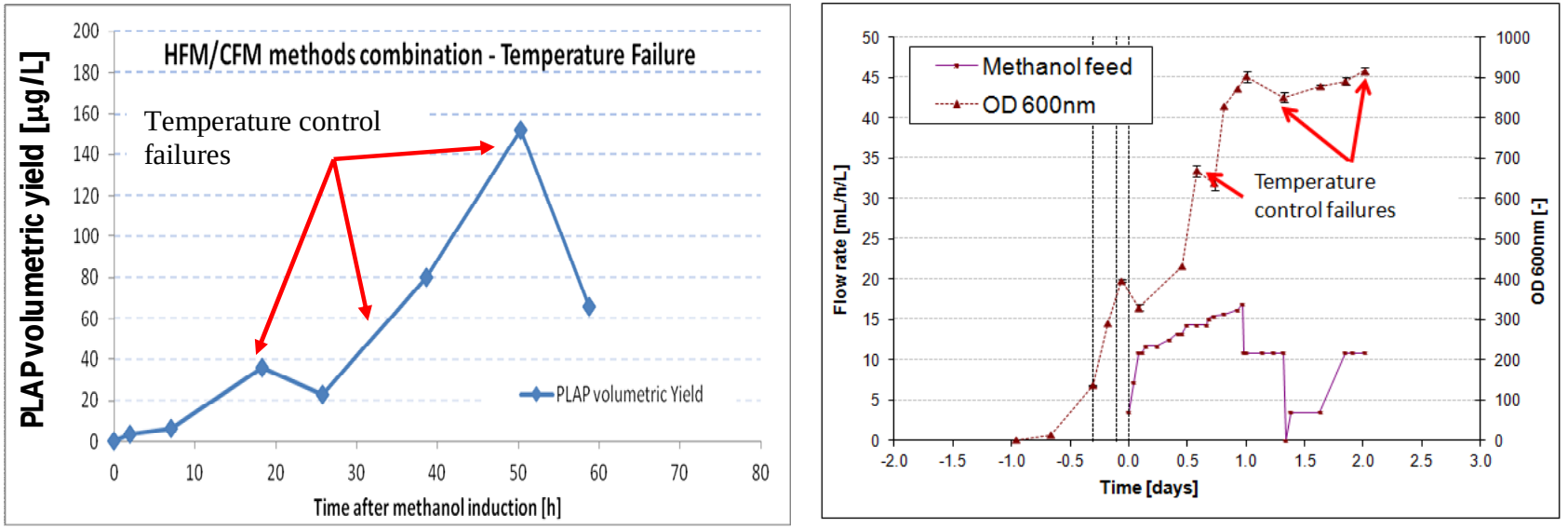


Figure 6.7: PLAP volumetric yield combined method at 1L scale – Temperature control failure
Curves generated from the fermentation of *Pichia pastoris* GS115 hPLAP Mut^{+} at 1L scale using HFM/CFM combination. The methanol feed rate was reduced in HFM after about 24 hours post-induction to generate the combination method HFM/CFM. Several temperature control failures resulted in a PLAP volumetric yield of 160 µg/L. The first temperature control failure had almost no effect. The second resulted in a methanol feed rate reduction of about 12 hours. The last temperature control failure (temperature reached over 50°C) resulted in high cell death rate and product loss with consequent fermentation ending. Time 0.0 is the time of methanol induction. The PLAP concentrations expressed are the sum of secreted and non-secreted PLAP.

6.4. Conclusions

At shake flask level, all four experimental strains were able to produce PLAP, which seems to have higher titres in αPLAP Mut^S clones. Concentrations were just under 40 µg/L for Mut^+ strains (better performance of hPLAP) and around 100 µg/L for Mut^S clones (better performance of αPLAP).

At bioreactor level, the new method (HFM/CFM combined) for higher protein production did not produce the desired outcome (increased productivity), even if a complicated fermentation did match CFM values. However, a few important points emerged from this research. First, it appeared that by increasing the methanol feed rate, cells reduce their protein production potential at the end of fermentation. Although higher total protein production was recorded in the first 48 hours after methanol induction, the higher cell density indicated lower productivity per cell titres. Finding the right balance of cell density and methanol feed is therefore a key parameter for the optimisation of *Pichia pastoris* fermentations. In fact, it was clear that cells grown with CFM had higher volumetric yield than cells grown with HFM. Due to vessel and method limitations, this last statement could change if longer fermentations will be performed with the HFM method with optimised methanol feeding rates. Indeed, cells grown with this method adapt faster to methanol and initiate protein production at an earlier point, allowing potential for higher total volumetric yield after 72 – 120 hours after methanol induction.

7. Discussion and conclusions

Pichia pastoris expression system is widely used for the production of heterologous proteins. However, processes need further optimisation in order to improve yields and cost-efficiency of this system. A few aspect of *Pichia pastoris* culture optimisation have been investigated within this project. The main findings, problems, and new knowledge acquired will be discussed in sections 7.1 to 7.5.

7.1. Objective 1: Investigate sensitivity of a DHAA-based assay for at-line methanol monitoring (Chapter 3)

It was shown that it is possible to measure residual methanol in biological samples with a quick a simple new assay. In fact, the reaction between DHAA and methanol could be quantified over time by spectrophotometrical analysis. It was possible to take the measure and have the results within 10-15 minutes from the moment the sample was taken from a bioreactor fermentation of *Pichia pastoris* cells. However, it was shown that concentrations below 10 g/L methanol had a low resolution, and for those below 3 g/L methanol the measure fell in the background noise of the instrument. On the other hand, for higher methanol concentrations, the assay worked well and offered high reproducibility. Therefore, even if the assay as it is was not suitable for measuring residual methanol during *Pichia pastoris* cultures, it could be used for qualitative analysis of presence/absence of methanol and for the detection of more concentrated methanol-containing samples in other industries, such as food (Georgia & Morales, 1926; Litchmann & Upton, 1972).

It was demonstrated that this assay works for the detection of methanol in biological samples. However, resolution for low methanol concentrations is poor, and therefore the use of this reaction for fermentation purposes is not practical. However, other application (e.g. qualitative analysis) are possible. Resolution for low methanol concentration can, eventually, be increased by testing new reaction condition. For instance, changing the reaction buffer or using different catalyser may offer a solution for the use of this novel assay to monitor methanol concentrations during *Pichia pastoris* fermentations.

7.2. Objective 2: Compare suitability of mechanistically distinct alkaline phosphatase activity assays for off-line PLAP monitoring (Chapter 3)

Two mechanistically different PLAP detection assays were compared in this work: chemiluminescent and colorimetric. From the obtained results optimisation of the colorimetric assay was more straightforward than the chemiluminescent one. However, the chemiluminescent assay offered a better sensitivity to low PLAP concentrations. On the other hand, even if reaction times were longer (no protein degradation was found within the reaction time) and the calibration curves were more laborious, the colorimetric assay was chosen to carry out all the PLAP assays in this project because of a better reproducibility.

The chemiluminescent assay still require better reproducibility, and therefore more work needs to be done on its optimisation. The colorimetric assay did work well for this work. However, long reaction times and multiple calibration curves are not ideal.

Perhaps some further work on it by modifying reagent/substrate ratios may solve those problems.

7.3. Objective 3: Construction and verification of P. pastoris PLAP variant overexpressor strains (Chapter 4)

Four novel experimental *Pichia pastoris* cell lines were created. The objective was to characterise the effect of methanol feed rates on productivity of trafficking variants of placental alkaline phosphatase (PLAP) in *Pichia pastoris* GS115. The gene was put under the control of the strong AOX1 promoter and two different secretion signals (human and yeast) were used to investigate differences in volumetric yield of PLAP. It was demonstrated that the PLAP gene, having the correct DNA sequence, was inserted into the genome of four new strains of *Pichia pastoris* GS115. It was shown that one Mut^+ and one Mut^S strain was created for each PLAP signal used. Even if the strains were successfully created and PLAP insertion confirmed, preliminary shake flasks experiments showed that the protein was not produced to high titres. In other words, it appeared that productivity levels were low compared to literature values. It was also noticed that Mut^S strains were capable of producing about 2.5-fold more PLAP than Mut^+ strains in shake flasks.

Protein production levels were lower than literature values. It would be advisable to re-construct the strains using a different selection method. In this work cells were selected purely by choosing the best performer in terms of growth in addition to a PCR analysis confirming PLAP integration into the *Pichia pastoris* genome.

7.4. Objective 4: Determine influence of methanol feed rate on biomass accumulation (Chapter 5)

It was showed in this project that by increasing the methanol feed-rate by almost 2-folds compared to the conventional *Pichia pastoris* fermentation method, it was possible to boost biomass formation after 24 hours from methanol induction by about 40%. Interestingly, those results were confirmed at both 1 and 20 litres scales. In addition, it emerged that the methanol induction methods (fast or slow) and the presence/absence of a transition phase (starvation) between the glycerol and methanol fed-batch phases had little or no influence on biomass generation after 24 hours in methanol.

Further possible improvements could be to test different parameters not widely studied in this work. For instance, more work on the induction method and oxygen supply can further improve cell viability. Similarly, different media could be tested (i.e. FM22 and d'Anjou) as well as different culture modes (i.e. Oxygen-Limited Fed-Batch - OLFB, Temperature-Limited Fed-Batch - TLFB).

7.5. Objective 5: Determine influence of methanol feed rate on specific yield of recombinant PLAP (Chapter 6)

From the results obtained in this project, it was clear that an increased methanol-flow-rate (HFM method) boosted biomass formation, whereas protein production was higher with conventional methanol supply (CFM method – 10.9 mL/L/h methanol). As a result, the two methods were combined with the objective to increase overall PLAP production. However, it was not the case for both *Pichia pastoris* GS115 Mut^+ αPLAP and *Pichia pastoris* GS115 Mut^+ hPLAP. However, production levels were 10 to 40-folds

lower than literature values (between 2 and 8 mg/L). It has to be said that PLAP activity data expressed in this work were the combination of secreted and retained PLAP, rather than secreted PLAP as usually shown in the literature. It was found that for the created strains secreted PLAP activity was near to zero, whereas the activity for non-secreted PLAP was higher. Even if the sequence of the secretion signal was as expected, protein trafficking and secretion within the cell has been impaired. Since it was not part of this work understating and investigating protein secretion in *Pichia pastoris*, no further work was done to explain this phenomenon, and all the PLAP data have been expressed as total combined PLAP concentration or yield. Even if combined PLAP activities were used, PLAP concentrations were lower compared to literature values.

All fermentation experiments were performed with *Pichia pastoris* Mut^+ hPLAP strain. It is possible that the human secretion signal affected the mRNA migration to the endoplasmic reticulum, reducing the protein production potential of the clones. However, no experiments have been performed to clarify this aspect. In addition, the created method was shorter (in terms of fermentation length) compared to the conventional method. As a result, it was possible that protein production was delayed within the cell, and by stopping the fermentation (reached maximal working volume within the vessel) at an earlier stage compared to the conventional methanol feed rate method, protein production could have had reached values similar or over the ones found in the literature. In fact, Nellaseth & Anderson (2013) stated that secreted alkaline phosphatase was only detected in the culture broth after 69 hours from induction.

Since, the assays used in this work to detect PLAP only measure active, correctly-folded recombinant protein, it is possible that the protein was produced but it remained,

for the majority, in a inactive form. In fact, Vanz et al. (2012) stated that under oxidative stress (methanol feed) cells responded with unfolded proteins, which were accumulated in the cell's ER.

As stated above, only one complete attempt to combine two different culture methods (HFM and CFM) was made. In addition, cells did exhibit low productivity from the early stages of this work. In addition, the literature stated that alkaline phosphatase is only detected in the culture broth after 69 hours from induction. As a result, it is advisable to extend fermentation times (i.e. by partly harvesting material from the vessel) post methanol induction to 80-100 hours instead of the 48-72 hours as is was done in this work.

References

Axelsson, H. (2000). *Encyclopedia of Bioprocess Technology*: *Fermentation, Biocatalysis, and Bioseparation*. *Wiley and Sons*, New York, Vol. 94.

Babel, W., Hofmann, K. H. (1982). The relation between the assimilation of methanol and glycerolin yeasts, *Arch Microbiol* , 132, 179-184.

Badrakhan, C.-D., Petrat, F., Holzhauser, M., Fuchs, A., Lomonosova, E. E., de Groot, H. (2004). The methanol method for the quantification of ascorbic acid and dehydroascorbic acid in biological samples. *J. Biochem. Biophys. Methods , 58*, 207-218.

Balamurugan, V., Reddy, G. R., Suryanarayana, V. V. S. (2007). *Pichia pastoris*: A notable heterologous expression system for the production of foreign proteins-vaccines. *Indian Journal of Biotechnology* , 6, 175-186.

Barnett, J. A., Payn, R. W., Yarrow, D. (1990). Yeasts: Characteristics and identification (Second ed.). Cambridge, *New York: Cambridge University Press*.

Barnett, J. A., Payn, R. W., Yarrow, D. (2000). Yeasts: Characteristics and identification (Third ed.). Cambridge, *New York: Cambridge University Press*.

Berger, J., Hauber, J., Hauber, R., Geiger, R., Cullen, B. R. (1988). Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene , 66*, 1-10.

Beynon, J. H., Fontaine, A. E., Lester, G. R. (1968). Mass spectrometry: the mass spectrum of methanol, Part I. Thermochemical information. *International Journal of Mass Spectrometry and Ion Physics* , 1(*1*), 1-24.

Boze, H., Laborde, C., Chemardin, P., Fabien, R., Venturin, C., Combarnous, Y., Moulin, G. (2001). High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*. *Process Biochem* , 36, 907-913.

Brady, C. P. S., Richard, L., Miles, A. P., Whitmore, M., Stowers, A. W. (2001). High-level production and purification of P30P2MSP119, an important vaccine antigen for malaria, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expression Purif.* , 23, 468-475.

Burrowes, O.-J., Diamond, G., Lee, T.-C. (2005). Recombinant expression of Pleurocidin cDNA using the *Pichia pastoris* expression system. *Journal of Biomedicine and Biotechnology , 2005(4)*, 374-384.

Chang, Y. C., Kao, W. C., Wang, W. Y., Wang, W. Y., Yang, R. B., Peck, K. (2009). Identification and characterisation of oligonucleotides that inhibit Toll-like receptor 2-associated immune responses. *The FASEB Journal* , 23(*9*), 3078-3088.

Charoenrat, T., Ketudat-Cairns, M., Stendahl-Andersen, H., Jahic, M., Enfors, S.-O. (2005). Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes. *Bioprocess Biosyst Eng* , 27, 399-406.

Charoenrat, T., Ketudat-Cairns, M., Jahic, M., Veide, A., Enfors, S.-O. (2006). Increased total air pressure versus oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process. *Biochem. Eng. J.* , 30, 205–211.

Charoenrat, T., Ketudat-Cairns, M., Enfors, S.-O., Jahic, M., Veide, A. (2006). Recovery of a recombinant β-glucosidase by expanded bed adsorption from *Pichia pastoris* high cell density culture broth. *J. Biotechnol.* , 100, 86-98.

Charoenrat, T., Ketudat-Cairns, M., Stendahl-Andersen, H., Jahic, M., Enfors, S.-O. (2005). Oxygen-limited fed-batch process: An alternative control for *Pichia pastoris* recombinant protein processes. *Bioprocess Biosyst. Eng.* , 27, 399-406.

Cos, O., Ramon, R., Montesinos, J. L., Valero, F. (2006). Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. *Microbial Cell Factories , 5 (17)*, 1-20.

Cos, O., Serrano, A., Montesinos, J. L., Ferrer, P., Cregg, J. M., Valero, F. (2005). Combined effect of methanol utilisation (Mut) phenotype and gene dosage on recombinant protein production in *Pichia pastoris* fed-batch cultures. *Journal of Biotechnology , 116*, 321-335.

Cregg, J. M. (1999). Expression in the methylotrophic yeast *Pichia pastoris*. In Gene Expression Systems: Using Nature for the Art of Expression. *Academic Press*: New York, 157-190.

Cregg, J. M., Vedvick, T. S., Raschke, W. C. (1993). Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology , 11*, 905-910.

Cullen, B. R., & Malim, M. H. (1992). Secreted placental alkaline phosphatase as a eukaryotic reporter gene. *Meth. Enzymol* , 216, 362-368.

Curvers, S., Linnemann, J., Klauser, T., Wandrey, C., Takors, R. (2001). Recombinant protein production with *Pichia pastoris* in continuous fermentation - Kinetic analysis of growth and product formation. *Chemie Ingenieur Technik , 12*, 1615-1621.

Damasceno, L., Pla, I., Chang, H.-J., Cohen, L., Ritter, G., Old, L. J., Batt, C. (2004). An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. *Protein Expression Purif.* , 37, 18-26.

De Schutter, K., Lin, Y.-C., Tiels, P., Van Hecke, A., Glinka, S., Weber-Lehmann, J. (2009). Genome sequence of the recombinant protein production host *Pichia pastoris. Nature Biotechnology , 27(6),* 561-566.

Duan, H., Umar, S., Hu, Y., Chen, J. (2009). Both the AOX1 promoter and the FLD1 promoter work together in a *Pichia pastoris* expression vector. *World Journal of Microbiology and Biotechnology , 25*, 1779-1783.

Dunn, W. A., Cregg, J. M., Kiel, J. A., Van der Klei, I. J., Oku, M., Sakai, Y. (2005). Pexophagy: The selective autophagy of peroxisomes. *Autophagy , 1 (2),* 75-83.

Egli, T., van Dijken, J. P., Veenhuis, M., Harder, W., Fietcher, A. (1980). Methanol metabolism in yeasts: Regulation of the synthesis of catabolic enzymes. *Arch Microbiol.* , 124, 115-122.

Fitzgerald, A., & Fitzgerald, B. (2004). Intellectual property in principle. *Sydney: Lawbook.*

Gancedo, J. M. (1998). Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* , 62, 334-361.

Gasser, B., Maurer, M., Rautio, J., Sauer, M., Bhattacharyya, A., Saloheimo, M. (2007). Monitoring of transcriptional regulation in *Pichia pastoris* under protein production conditions. *BMC Genomics , 179(8),* 1-18.

Gellissen, G. (2000). Heterologous protein production in methylotrophic yeasts. *Appl Microbiol Biotechnol* , 54, 741–750.

Georgia, F. R. & Morales, R. (1926). Detection of Methanol in Alcoholic Beverages. *Ind. Eng. Chem.* , 18(*3*), 304-306.

Guarna, M. M., Lesnicki, G. J., Ram, B. M., Robinson, J., Radziminski, C. Z., Hasenwinkle, D., Boraston, A., Jervis, E., MacGillivray, R. T. A., Turner, R. F. B., Kilburn, D. G. (1997). On-line monitoring and control of methanol concentration in shake-flask cultures of *Pichia pastoris*. *Biotechnol. Bioeng.* , 56(3), 279-286.

Guo, Y., Lu, F., Zhao, H., Tang, Y., Lu, Z. (2009). Cloning and heterologous expression of glucose oxidase gene from Aspergillus niger Z-25 in *Pichia pastoris*. *Appl. Biochem. Biotechnol. , 1-12*.

Hartner, F. S., & Glieder, A. (2006). Regulation of methanol utilisation pathway genes in yeasts. Microbial Cell Factories , *5 (39)*.

Hartner, F. S., Ruth, C., Langenegger, D., Johnson, S. N., Hyka, P., Lin-Cereghino, G. P. (2008). Promoter library designed for fine-tuned gene expression in *Pichia pastoris*. Nucleic Acids Research , *36(12)*, 1-15.

He, J., Yu, B., Zhang, K., Ding, X., Chen, D. (2009). Expression of endo-1,4-beta-xylanase from Trichoderma reesei in *Pichia pastoris* and functional characterization of the produced enzyme. *BMC Biotechnology , 56(9),* 1-10.

Heimo, H., Palmu, K., Suominen, I. (1997). Human placental alkaline phosphatase: Expression in *Pichia pastoris*, purification and characterisation of the enzyme. *Protein expression and purification,* 12, 85-92.

Hellwig, S., Emde, F., Raven, H. P. G., Henke, M., van der Logt, P., Fischer, R. (2001). Analysis of single-chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations. *Biotechnol. Bioeng.* , 74(4), 344-352.

Hollenberg, C. P., Gellissen, G. (1997). Production of recombinant proteins by methylotrophic yeasts. *Current Opinion in Biotechnology , 8,* 554-560.

Holmes, W. J., Darby, R. A., Wilks, M. D., Smith, R., Bill, R. M. (2009). Developing a scalable model of recombinant protein yield from *Pichia pastoris*: the influence of culture conditions, biomass and induction regime. *Microbial Cell Factories , 8(35),* 1-14.

Hong, F., Meinhander, N. Q., Jonsson, L. J. (2002). Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnol. Bioeng.* , 79, 438-449.

Hong, I.-P., Lee, S.-J., Kim, Y.-S., Choi, S.-G. (2007). Recombinant expression of human cathelicidin (hCAP18/LL-37) in *Pichia pastoris. Biotechnology Letters , 29,* 73-78.

Houard, S., Heinderyckx, M., Bollen, A. (2002). Engineering of non-conventional yeasts for efficient synthesis of macromolecules: the methylotrophic genera. *Biochim Biophys Acta* , 1555, 44-47.

Hribar, G., Smilovic, V., Zupan, A. L., Gaberc-Porekar, V. (2008). β-lactamase reporter system for selecting high-producing yeast clones. *BioTechniques , 44,* 477-484.

Hung, H-C. & Chang, G.-G. (2001). Multiple Unfolding Intermediates of Human Placental Alkaline Phosphatase in Equilibrium Urea Denaturation. *Biophysical Journal*, 81, 3456-3471.

Inan, M. (2000). Studies on the alcohol oxydase (AOX1) promoter of *Pichia pastoris*. In *PhD thesis* University of Nebraska, USA, Department of Food Science and Technology.

Inan, M., & Meagher, M. M. (2001). The effect of ethanol and acetate on protein expression in *Pichia pastoris. Journal of Bioscience and Bioengineering , 92(4),* 337-341.

Inan, M., Meagher, M. M., Benson, A. K. (2004). Alcohol oxydase I regulatory sequences for heterologous gene expression in Yeast. US6699691, The board of Regents of the University of Nebraska, USA.

Jahic, M., Rotticci-Mulder, J.C., Martinelle, M., Hult, K., Enfors, S-O. (2002). Modelling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein. *Bioprocess Biosyst Eng*, 24, 385-393.

Jahic, M., Veide, A., Charoenrat, T., Teeri, T., Enfors, S.-O. (2006). Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. *Biotechnol. Prog. , 22*, 1465-1473.

Jahic, M., Wallberg, F., Bollok, M., Garcia, P., Enfors, S.-O. (2003). Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microbial Cell Factories* , 2:6.

Janowicz, Z. A., Eckart, M. R., Drewke, C., Roggenkamp, R. O., Hollenberg, C. P., Maat, J., Ledeboer, A. M., Visser, C., Verrips, C. T. (1985). Cloning and characterization oft he DAS gene encoding the major methanol assimilatory enzyme from the methylotrophic yeast *Hansenula polymorpha*. *Nucleic Acids Res* , 13, 3043-3062.

Jungo, C., Marison, I., Von Stockar, U. (2007). Mixed feeds of glycerol and methanol can improve the performance of *Pichia pastoris* cultures: A quantitative study based on concentration gradients in transient continuous cultures. *Journal of Biotechnology , 128*, 824-837.

Jungo, C., Schenk, J., Pasquier, M., Marison, I. W., Von Stockar, U. (2007). A quantitative analysis of the benefits of mixed feeds of Sorbitol and methanol for the production of recombinant avidin with *Pichia pastoris*. *Journal of Biotechnology , 131*, 57-66.

Khatri, N. K. (2011). Optimisation of recombinant protein production in *P. pastoris*. Single-chain antibody fragment model protein. University of Oulu, Faculty of Technology, C 393.

Kim, S. J., Lee, J. A., Won, K., Kim, Y. H., Song, B. K. (2009). Functional expression of Coprinus cinereus peroxidise in *Pichia pastoris*. *Process Biochemistry* , 44, 731-735.

Koch, V., Ruffer, H. M., Schugerl, K., Innertsberger, E., Menzel, H. Weis, J. (1995). Effect of antifoam agents on the medium and microbial cell properties and process performance in small and large reactors. *Process Biochem* , 30, 435-446.

Krainer, F. W., Dietzsch, C., Hajek, T., Herwig, C., Spadiut, O., Glieder, A. (2012).Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway. *Microbial Cell Factories* , 11:22.

Kumagai, M. H., Sverlow, G. G. (1997). *Pichia pastoris* alcohol oxidase ZZA1 and ZZA2 regulatory regions for heterologous gene expression. US5641661, Biosource Technologies, Inc., USA.

Li, H., Wang, D., Xu, A., Li, S., Jin, S., Wu, D. (2009). High level expression and purification of active recombinant human interleukin-8 in *Pichia pastoris. Protein Expression and Purification , 68(1),* 60-64.

Li, Z. J., Xiong, F., Lin, Q. S., d'Anjou, M. Daugulis, A. J., Yang, D. S. C., Hew, C. L. (2001). Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. *Protein Express Purif* , 21, 438-445.

Lin-Cereghino, J., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris. FEMS Microbiology Reviews , 24*, 45-66.

Lin-Cereghino, G. P., Godfrey, L., de la Cruz, B. J., Johnson, S., Khuongsathiene, S., Tolstorukov, I., Yan, M., Lin-Cereghino, J., Veenhuis, M., Subramani, S., Cregg, J. M. (2006). Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in *Pichia pastoris*. *Mol Cell Biol* , 26, 883-897.

Ling, Y. L., Ithoi, I., Yik, F. M. (2010). Optimization for high-level expression in *Pichia pastoris* and purification of truncated and fell length recombinant SAG2 of *Toxoplasma gondii* for diagnostic use. *Southeast Asian J Trop Med Public Health* , 41(*3*), 507-513.

Litchmann, M. A., & Upton, R. P. (1972). Gas chromatographic determination of residual methanol in food additives. *Anal. Chem. ,* 44(*8*), 1495-1497.

Lombardi, A., Bursomanno, S., Lopardo, T., Traini, R., Colombatti, M., Ippoliti, R., Flavell, D. J., Flavell, S. U., Ceriotti, A., Fabbrini, M. S. (2010). *Pichia pastoris* as a host for secretion of toxic saporin chimeras. *The FASEB Journal*, 24, 253-265.

Lucarelli, P., Scacchi, R., Corbo R.M., Benincasa, A., Palmariono, R. (1982). Human placental alkaline phosphatase electrophoretic alleles: Quantitative studies. *Am J Hum Genet.*. 34(*2*), 331-336.

Macauley-Patrick, S., Fazenda, M. L., McNeil, B., Harvey, L. M. (2005). Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* , 22, 249-270.

Mattanovich, D., Callewaert, N., Rouze, P., Lin, Y.-C., Graf, A., Redl, A. (2009). Open access to sequence: Browsing the *Pichia pastoris* genome. *Microbial Cell Factories , 8:53*, 1-4.

Mi-Jin, K., Kim, S.-H., Lee, J. H., Seo, J.-H., Lee, J.-H., Kim, J.-H. (2008). High-level secretory expression of human Procarboxypeptidase B by fed-batch cultivation of *Pichia pastoris* and its partial characterization. *Journal of Microbiol. Biotechnol. , 18(12),* 1938-1944.

Murray, W. D., Duff, S. J., Beveridge, T. J. (1990). Catabolite inactivation in the methylotrophic yeast *Pichia pastoris*. *Appl Environ Microbiol* , 56, 2378-2383.

Nallaseth, F. S. & Anderson, S. (2013). A screen for over-secretion of proteins by yeast based on a dual component cellular phosphatase and immune-chromogenic stain for exported bacterial alkaline phosphatase reporter. *Microbial Cell Factories* , 12:36.

Nakagawa, T., Mizumura, T., Mukaiyama, H., Miyaji, T., Yurimoto, H., Kato, N., Sakay, Y., Tomizuka, N. (2002). Physiological role of the second alcohol oxidase gene MOD2 in the methylotrophic growth of *Pichia methanolica*. *Yeast* , 19, 1067-1073.

Ohi, H., Miura, M., Hiramatsu, R., Ohmura, T. (1994). The positive and negative cis-acting elements for methanol regulation in the *Pichia pastoris* AOX2 gene. *Mol Gen Genet*, 243, 489-499.

Orman, M. A., Calik, P., Ozdamar, T. H. (2009). The influence of carbon sources on recombinant-human-growth-hormone production by *Pichia pastoris* is dependent on phenotype: a comparison of Mut^S and Mut^+ strains. *Biotechnol. Appl. Biochem. , 52,* 245-255.

Papanikou, E., & Glick, B. S. (2009). The yeast Golgi apparatus: Insights and mysteries. *FEBS Letters , 583,* 3746-3751.

Pla, I. A., Damasceno, L. M., Vannelli, T., Ritter, G., Batt, C. A., Shuler, M. L. (2006). Evaluation of Mut^+ and Mut^S *Pichia pastoris* phenotypes for high level extracellular scFv expression under feedback control of the methanol concentration. *Biotechnology Progress , 22,* 881-888.

Ramchuran, S. O. M. B., Holst, O., Karlsson, E. N. (2005). The methylotrophic yeast *Pichia pastoris* as a host for the expression and production of thermostable xylanase from the bacterium *Rhodothermus marinus*. *FEMS Yeast Res.* , 5, 839-850.

Ratledge, C. (1991). Yeast physiology - a micro-synopsis. *Bioprocess Engineering , 6,* 195-203.

Resina, D., Cos, O., Ferrer, P., Valero, F. (2005). Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen source-regulated FLD1 promoter. *Biotechnology and Bioengineering , 91(6),* 760-767.

Ruottinen, M., Bollok, M., Kogler, M., Neubauer, A., Krause, M., Hamalainen, E.-R., (2008). Improved production of human type II procollagen in the yeast *Pichia pastoris* in shake flasks by a wireless-controlled fed-batch system. *BMC Biotechnology , 33(8),* 1-12.

Sakay, Y., Nakagawa, T., Shimase, M., Kato, N. (1998). Regulation and physiological role of the DAS1 gene, encoding dihydroxyacetone synthase, in the methylotrophic yeast *Candida boidinii*. *J. Bacteriol* , 180, 5885-5890.

Schenk, J., Marison, J. W., von Stockar, U. (2007). A simple method to monitor and control methanol feeding of *Pichia pastoris* fermentations using mid-IR spectroscopy. *Journal of Biotechnology , 128,* 344-353.

Schlaeger, E. J., Kitas, E. A., Dorn, A. (2003). SEAP expression in transiently transfected mammalian cells grown in serum-free suspension culture. *Cytotechnology ,* 42(1), 47-55.

Scorer, C. A., Clare, J. J., McCombie, W. R., Romanos, M. A., Sreekrishna, K. (1994). Rapid selection using G148 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Nature Biotechnology , 12,* 181-184.

Sivaprakasam, S., Schuler, M. M., Hama, A., Hughes, K.-M., Marison, I. W. (2011). Biocalorimetry as a process analytical technology process analyser; robust in-line monitoring and control of aerobic fed-batch cultures of crabtree-negative yeast cells. *Journal of Thermal Analysis and Calorimetry ,* 104*(1),* 75-85.

Sreekrishna, K., Kropp, K. (1996). *Pichia pastoris*. In: Wolf, K. (Ed.), Non conventional yeasts in biotechnology. *Springer,* Berlin, 203-252.

Sreekrishna, K., Brankamp, R. G., Kropp, K., Blankenship, D. T., Tsay, J., Smith, P. L., Wierschke, J. D., Subramanian, A., Birkenberger, L- A. (1997). Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* , 190, 55-62.

Stratton, J., Chiruvolu, V., Meagher, M. (1998). High cell-density fermentation. *Methods in Molecular Biology , 103:Pichia Protocols,* 107-120.

Suh, S.-O., Nguyen, N. H., Blackwell, M. (2005). Nine new *Candida* species near *C. membranifaciens* isolated from insects. *Mycological Research , 109 (9),* 1045-1056.

Szamecz, B., Urban, G., Rubiera, R., Kucsera, J., Dorgai, L. (2005). Identification of four alcohol oxidases from methylotrophic yeast. *Yeast* , 22, 669-676.

Teng, E. L. W. & Samyudia, Y. (2011). Nonlinear Model-Based Control of Non-Ideally Mixed Fermentation Processes. *2010 International Conference on Biology, Environment and Chemistry, IPCBEE,* vol. 1, 26-33.

Thorpe, E. D., d'Anjou, M. C., Daugulis, A. J. (1999). Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. *Biotechnol Letters ,* 21, 669-672.

Trentmann, O., Khatri, N. K., Hoffmann, F. (2004). Reduced oxygen supply increases process stability and product yield with recombinant *Pichia pastoris*. *Biotechnol. Prog*., 20, 1766-1775.

Trihn, L. B., Phue, J. N., Shiloach, J. (2003). Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from *Pichia pastoris*. *Biotechnol Bioeng* , 82(*4*), 438-444.

van Dijken, J. P., Otto, R., Harder, W. (1976). Growth of *Hansenula polymorpha* in a methanol-limited chemostat. *Arch Microbiol,* 111, 137-144.

Van der Klei, I. J., Yurimoto, H., Sakai, Y., Veenhuis, M. (2006). The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochimica et biophysica Acta , 1763,* 1453-1462.

Van Urk, H., Voll, W. S. L., Scheffers, A., Van Dijken, J. P. (1990). Transient-State Analysis of Metabolic Fluxes in Crabtree-Positive and Crabtree-negative Yeasts. *Appl. Environm. Microbiol.* , 56*(1),* 281.

Vanz, A. L., Lunsdorf, H., Adnan, A., Nimtz, M., Gurramkonda, C., Khanna, N., Rinas, U. (2012). Physiological response of *Pichia pastoris* GS115 to methanol-induced high level production of the Hepatitis B surface antigen: catabolic adaptation, stress responses, and autophagic processes. *Microbial Cell Factories* , 11, 103.

Vervecken, W., Kaigorodov, V., Callewaert, N., Geysens, S., De Vusser, K., Contreras, R. (2004). In vivo synthesis of mammalian-like, hybrid-type N-Glycans in *Pichia pastoris*. *Applied and Environmental Microbiology , 70(5),* 2639-2646.

Wagner, L. W., Matheson, N. H., Heisey, R. F., Scheneider, K. (1997). Use of a silicon tubing sensor to control methanol concentration during fed batch fermentation of *Pichia pastoris*. *Biotechnol. Tech.* , 11, 791-795.

Waters, M. G., Evans, E. A., Blobel, G. (1988). Prepro-alpha-factor has a cleavable signal sequence. *J Biol Chem.* , 263(*13*), 6209-14.

Wegner, E. H. (1983). Biochemical conversions by yeast fermentation at high cell densities. Volume 316164. Edited by *Company PP*. Bartlesville, Oklahoma. U.S. Patent 110,457

Wildt, S., & Gerngross, T. U. (2005). The humanization of N-glycisylation pathways in yeast. *Nature , 3(Feb),* 119-128.

Wu, J. M., Wang, A. Y., Fu, W. C. (2012). Lower Temperature Cultures Enlarge the Effects of Vitreoscilla Hemoglobin Expression on Recombinant *Pichia pastoris*. *Int. J. Mol. Sci.* , 12, 13212-13226.

Xuan, Y., Zhou, X., Zhang, W., Zhang, X., Song, Z., Zhang, Y. (2009). An upstream activation sequence controls the expression of AOX1 gene in *Pichia pastoris*. *Yeast research* , 1-12.

Yang, T. T., Sinai, P., Kitts, P. A., Kain, S. R. (1997). Quantification of Gene Expression with a Secreted Alkaline Phosphatase Reporter System. *BioTechniques* , 23, 1110-1114.

Yurimoto, H. (2009). Molecular basis of methanol-inducible gene expression and its application in the methylotrophic yeast *Candida boidinii*. *Biosci. Biotechnol. Biochem. , 73 (4),* 793-800.

Yurimoto, H., & Sakai, Y. (2009). Methanol-inducible gene expression and heterologous protein production in the methylotrophic yeast *Candida boidinii*. *Biotechnology and Applied Biochemistry , 53*, 85-92.

Zhang, W., Bevins, M. A., Plantz, B. A., Smith, L. A., (2000). Modelling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of Botulinum neurotoxin, serotype A. *Papers in Biotechnology*. Paper 18

Zhang, F., Wolff, M. W., Williams, D., Busch, K., Lang, S. C., Murhammer, D. W., Linhardt, R. J. (2000). Affinity purification of secreted alkaline phosphatase produced by Baculovirus expression vector system. *Applied Biochemistry and Biotechnology* , 90, 125-136.

Zhang, W., Potter, K. J. H., Plantz, B. A., Schlegel, V. L., Smith, L. A., Meagher, M. M. (2003). *Pichia pastoris* fermentation with mixed-feed of glycerol and methanol: growth kinetics and production improvement. *Papers in Biochemical Engineering , Chemical and Biomolecular Engineering Research and Publications*.

Zhang, J.-G., Wang, X.-D., Zhang, J.-N., Wei, D.-Z. (2008). Oxygen vectors used for S-Adenosylmethionine production in recombinant *Pichia pastoris* with sorbitol as supplemental carbon source. *Journal of Bioscience and Bioengineering , 105(4),* 335-340.

Zhao, H. L., Xue, C., Wang, Y., Yao, X. Q., Liu, Z. M. (2008). Increasing the cell viability and heterologous protein expression of *Pichia pastoris* mutant deficient in PMR1 gene by culture condition optimization. *Appl. Microbiol. Biotechnol. , 81,* 235-241.

Zhu, T., Guo, M., Sun, C., Qian, J., Zhuang, Y., Chu, J. (2009). A systematical investigation on the genetic stability of multi-copy *Pichia pastoris* strains. *Biotechnology Letters , 31,* 679-684.